

UTILITY PATENT APPLICATION

AMINO POLYOL AMINE OXIDASE POLYNUCLEOTIDES AND RELATED  
POLYPEPTIDES AND METHODS OF USE

Inventors:

Jonathan P. Duvick  
a United States citizen residing at,  
1707 38<sup>th</sup> Street, Des Moines, Iowa 50310

Jacob T. Gilliam  
a United States citizen residing at,  
1004 Meadow Drive, Norwalk, Iowa 50211

Joyce R. Maddox  
a United States citizen residing at,  
1312 N. 126<sup>th</sup> Street, Omaha, NE 68154

Oswald R. Crasta  
a citizen of India residing at,  
95-2C Florence Road, Branford, Connecticut CT06405

Otto Folkerts  
a citizen of the Netherlands residing at,  
29 Arrowhead Drive, Guilford, Connecticut CT06437

5

**Technical Field**

The present invention relates generally to the detection and isolation of fumonisin and AP1 degrading enzymes and to compositions and methods for the *in vivo* detoxification or degradation of fumonisin or its hydrolysis product AP1. This method has broad application in agricultural biotechnology and crop agriculture and in the improvement of food grain quality.

**Cross Reference To Related Application**

15

This application claims the benefit of U.S. Provisional Application No. 60/092,936 filed July, 15 1998 and is a Continuation of U.S. Application No. 09/352,168 filed July 12, 1999, and they are hereby incorporated by reference.

**Background of the Invention**

Fungal diseases are common problems in crop agriculture. Many strides have been made against plant diseases as exemplified by the use of hybrid plants, pesticides and improved agricultural practices. However, as any grower or home gardener can attest, the problems of fungal plant disease continue to cause difficulties in plant cultivation. Thus, there is a continuing need for new methods and materials for solving the problems caused by fungal diseases of plants.

These problems can be met through a variety of approaches. For example, the infectious organisms can be controlled through the use of agents that are selectively biocidal for the pathogens. Another method is interference with the mechanism by which the pathogen invades the host crop plant. Yet another method, in the case of pathogens that cause crop losses, is interference with the mechanism by which the pathogen causes injury to the host crop plant. Still another method, in the case of pathogens that produce toxins that are undesirable to mammals or other animals that feed on the crop plants, is

interference with toxin production, storage, or activity. This invention falls into the latter two categories.

Since their discovery and structural elucidation in 1988 (Bezuidenhout *et al.*, *Journal Chem Soc, Chem Commun* 1988: 743-745 (1988)), fumonisins have been  
5 recognized as a potentially serious problem in maize-fed livestock. They are linked to several animal toxicoses including leukoencephalomalacia (Marasas, *et al.*, *Onderstepoort Journal of Veterinary Research* 55: 197-204 (1988); Wilson, *et al.*, *American Association of Veterinary Laboratory Diagnosticians: Abstracts 33rd Annual Meeting*, Denver, Colorado, October 7-9, 1990, Madison, Wisconsin, USA) and porcine pulmonary edema  
10 (Colvin, *et al.*, *Mycopathologia* 117: 79-82 (1992)). Fumonisins are also suspected carcinogens (Geary W (1971) *Coord Chem Rev* 7: 81; Gelderblom, *et al.*, *Carcinogenesis* 12: 1247-1251 (1991); Gelderblom, *et al.*, *Carcinogenesis* 13: 433-437 (1992)). *Fusarium* isolates in section *Liseola* produce fumonisins in culture at levels from 2 to >4000 ppm (Leslie, *et al.*, *Phytopathology* 82: 341-345 (1992)). Isolates from maize (predominantly  
15 mating population A) are among the highest producers of fumonisin. (Leslie *et al.*, *supra*). Fumonisin levels detected in field-grown maize have fluctuated widely depending on location and growing season, but both preharvest and postharvest surveys of field maize have indicated that the potential for high levels of fumonisins exists (Murphy, *et al.*, *J Agr Food Chem* 41: 263-266 (1993)). Surveys of food and feed products have also detected  
20 fumonisin (Holcomb, *et al.*, *J Agr Food Chem* 41: 764-767 (1993); Hopmans, *et al.*, *J Agr Food Chem* 41: 1655-1658 (1993); Sydenham, *et al.*, *J Agr Food Chem* 39: 2014-2018 (1991)). The etiology of *Fusarium* ear mold is poorly understood, although physical damage to the ear and certain environmental conditions can contribute to its occurrence (Nelson, *Mycopathologia* 117: 29-36 (1992)). *Fusarium* can be isolated from most field  
25 grown maize, even when no visible mold is present. The relationship between seedling infection and stalk and ear diseases caused by *Fusarium* is not clear. Genetic resistance to visible kernel mold has been identified (Gendloff, *et al.*, *Phytopathology* 76: 684-688 (1986); Holley, *et al.*, *Plant Dis* 73: 578-580 (1989)), but the relationship to visible mold to fumonisin production has yet to be elucidated.

30 Fumonisins have been shown in *in vitro* mammalian cell studies to inhibit sphingolipid biosynthesis through inhibition of the enzyme sphingosine N-acetyl transferase, resulting in the accumulation of the precursor sphinganine (Norred, *et al.*,

*Mycopathologia* 117: 73-78 (1992); Wang, *et al.*, *Biol Chem* 266: 14486 (1991); Yoo, *et al.*, *Toxicol Appl Pharmacol* 114: 9-15 (1992); Nelson, *et al.*, *Annu Rev Phytopathol* 31:233-252 (1993)). It is likely that inhibition of this pathway accounts for at least some of fumonisin's toxicity, and support for this comes from measures of sphinganine: sphingosine ratios in animals fed purified fumonisin (Wang, *et al.*, *J Nutr* 122: 1706-1716 (1992)). Fumonisin also affect plant cell growth (Abbas, *et al.*, *Weed Technol* 6: 548-552 (1992); Vanasch, *et al.*, *Phytopathology* 82: 1330-1332 (1992); Vesonder, *et al.*, *Arch Environ Contam Toxicol* 23: 464-467 (1992)). Kuti *et al.*, (Abstract, Annual Meeting American Phytopathological Society, Memphis, TN: APS Press 1993) reported on the ability of exogenously added fumonisins to accelerate disease development and increase sporulation of *Fusarium moniliforme* and *F. oxysporum* on tomato.

Enzymes that degrade the fungal toxin fumonisin to its de-esterified form (e.g. AP1 from FB1) have been identified in US patent no. 5,716,820, issued February 10, 1998 US patent no. 5,792,931 issued August 11, 1998; and pending US applications nos. 08/888,950 and 08/888,949, both filed July 7, 1997, and all hereby incorporated by reference. It is understood that AP1 as used here is to designate the hydrolyzed form of any fumonisin, FB1, FB2, FB3, FB4, or any other AP1-like compounds, including synthetically produced AP1 like compounds, that contain a C-2 or C-1 amine group and one or more adjacent hydroxyl groups. Plants expressing a fumonisin esterase enzyme, infected by fumonisin producing fungus, and tested for fumonisin and AP1 were found to have low levels of fumonisin but high levels of AP1. AP1 is less toxic than fumonisin to plants and probably also to animals but contamination with AP1 is still a concern. The preferred result would be complete detoxification of fumonisin to a non-toxic form. Therefore enzymes capable of degrading AP1 are necessary for the further detoxification of fumonisin. The present invention provides newly discovered polynucleotides and related polypeptides of amino polyol amine oxidase (abbreviated APAO, formerly known as AP1 catabolase, US patent no. 5,716,820, *supra*; US patent no. 5,792,931, *supra*, and pending US applications nos. 08/888,950 and 08/888,949, *supra*; trAPAO is the abbreviation for a truncated, but still functional APAO), capable of oxidatively deaminating the AP1 to a compound identified as the 2-oxo derivative of AP1 or its cyclic ketal form (abbreviated as 2-OP, formerly called AP1-N1, US patent no. 5,716,820, *supra*; US patent no. 5,792,931, *supra*; pending US applications nos. 08/888,950 and 08/888,949,

*supra*), isolated from *Exophiala spinifera*, ATCC 74269. The partially purified APAO enzyme from *Exophiala spinifera* has little or no activity on intact FB1, a form of fumonisin. However, recombinant APAO enzyme from *Exophiala spinifera*, expressed in *E. coli*, has significant but reduced activity on intact FB1 and other B-series fumonisins.

5 APAO or trAPAO thus could potentially be used without fumonisin esterase since the amine group is the major target for detoxification. Alternatively, fumonisin esterase and APAO (or trAPAO) can be used together for degrading toxins.

APAO is a type of flavin amine oxidase (EC 1.4.3.4, enzyme class nomenclature, see *Enzyme Nomenclature 1992*, Recommendations of the Nomenclature Committee of the

10 IUBMB on the Nomenclature and Classification of Enzymes, Academic Press, Inc. (1992)). Flavin amine oxidases are known in mammals as monoamine oxidases, where they participate in the conversion of amines involved in neuronal function. A prokaryotic flavin amine oxidase that deaminates putrescine has been described (Ishizuka *et al.*, *J. Gen Microbiol.* 139:425-432 (1993)). A single fungal gene, from *Aspergillus niger* has been

15 cloned (Schilling *et al.*, *Mol Gen Genet.* 247:430-438 (1995)). It deaminates a variety of alkyl and aryl amines, but when tested for its ability to oxidize AP1, was found to not contain AP1 oxidizing activity.

The toxicity of fumonisins and their potential widespread occurrence in food and feed makes it imperative to find detoxification or elimination strategies to remove the

20 compound from the food chain.

### Summary of the Invention

The present invention provides polynucleotides, related polypeptides and all conservatively modified variants of a newly discovered APAO. The nucleotide sequence of

25 the APAO comprises the sequence found in SEQ ID NOS: 5, 10, and 22. SEQ ID NO: 5 contains the nucleotide sequence of trAPAO, SEQ ID NO: 10 contains the nucleotide sequence of trAPAO with an additional lysine and SEQ ID NO: 22 contains the full length nucleotide sequence of APAO. For expression in a plant, the nucleotide sequence of APAO or trAPAO is fused to a plant signal sequence. Preferred plant signal sequences are

30 signal sequences which target the apoplast or a peroxisome. Other signal sequences can also be used, depending on requirements, including mitochondrial or plastidic. It is an

object of the present invention to provide transgenic plants and plant cells comprising the nucleic acids of the present invention.

Therefore, in one aspect, the present invention relates to an isolated nucleic acid comprising an isolated polynucleotide sequence encoding an APAO enzyme. In a further aspect, the present invention is selected from: (a) an isolated polynucleotide encoding a polypeptide of the present invention; (b) a polynucleotide having at least 70% identity to a polynucleotide of the present invention; (c) a polynucleotide comprising at least 25 nucleotides in length which hybridizes under high stringency conditions to a polynucleotide of the present invention; (d) a polynucleotide comprising a polynucleotide of the present invention; and (e) a polynucleotide which is complementary to the polynucleotide of (a) to (f).

Additional polynucleotides of the present invention include an APAO enzyme fused to a fumonisin esterase. The fumonisin esterase is preferably ESP1 or BEST1.

In another aspect, the present invention relates to a recombinant expression cassette comprising a nucleic acid as described, *supra*. Additionally, the present invention relates to a vector containing the recombinant expression cassette. Further, the vector containing the recombinant expression cassette can facilitate the transcription and translated of the nucleic acid in a host cell. The present invention also relates to the host cells able to express the polynucleotide of the present invention. A number of host cells could be used, such as but not limited to, microbial, mammalian, plant, or insect.

In yet another embodiment, the present invention is directed to a transgenic plant or plant cells, containing the nucleic acids of the present invention. Preferred plants containing the polynucleotides of the present invention include but are not limited to maize, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, tomato, and millet. In another embodiment, the transgenic plant is a maize plant or plant cells. In yet another embodiment are the seeds from the transgenic plant.

This invention also provides an isolated polypeptide comprising (a) a polypeptide comprising at least 70% sequence identity to a polypeptide of the present invention; (b) a polypeptide encoded by a nucleic acid of the present invention; and (c) a polypeptide characterized by a polypeptide selected from SEQ ID NOS: 6, 11, 23, 36, 38, 40, 42, 44, and 46.

Another embodiment of the subject invention comprises a method of reducing pathogenicity of a fungus producing fumonisin or a structurally related mycotoxin by transferring to a plant the nucleic acids of the present invention either by themselves or in combination with a nucleic acid coding for a fumonisin esterase. In addition, two plants,  
5 one of which is transformed with an APAO of the present invention and the other transformed with a fumonisin esterase, can be crossed to produce a plant expressing both fumonisin esterase and APAO.

This invention further provides methods of degrading a fumonisin, a fumonisin breakdown product, a structurally related mycotoxin or a breakdown product of a structurally related mycotoxin, comprising the step of reacting the mycotoxin with the  
10 degradative enzymes of the present invention. Additionally, fumonisins can be degraded by application of both fumonisin esterase enzymes and APAO enzyme. The mycotoxins can be degraded in harvested grain, during the processing of harvested grain, in animal feed, or in plant tissue as, for example, during the use of the plant for silage or as a spray  
15 on grain, fruit or vegetables.

Another embodiment of the subject invention is a host cell stably transformed by a polynucleotide construct as described above, and a method of making a polypeptide of a recombinant gene comprising:

- a) providing a population of these host cells; and
- 20 b) growing the population of cells under conditions whereby the polypeptide encoded by the coding sequence of the expression cassette is expressed;
- c) isolating the resulting polypeptide.

A number of expression systems using the said host cells could be used, such as but not limited to, microbial, mammalian, plant, or insect. Alternatively, the fumonisin  
25 degrading enzymes can be isolated and purified from the seeds or plant parts of a plant expressing the said enzyme.

The polynucleotides of the present invention can also be used as a selectable marker for plant transformation. By transforming plant cells with an expression cassette containing the polynucleotide of the present invention and then placing the plant cells on  
30 media containing AP1 or a phytotoxic analog, only the plant cells expressing the polynucleotide of the present invention would survive.

Another embodiment of the present invention is the use of the enzyme fumonisin esterase and APAO by themselves or in combination as reagents for detecting fumonisin and structurally related toxins.

## 5 Detailed Description of the Invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting. The following is presented by way of illustration and is not intended to limit the scope of the invention.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of botany, microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, J. H. Langenheim and K. V. Thimann, *Botany: Plant Biology and Its Relation to Human Affairs* (1982) John Wiley; *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 1 (I. K. Vasil, ed. 1984); R. V. Stanier, J. L. Ingraham, M. L. Wheelis, and P. R. Painter, *The Microbial World*, (1986) 5th Ed., Prentice-Hall; O. D. Dhringra and J. B. Sinclair, *Basic Plant Pathology Methods*, (1985) CRC Press; Maniatis, Fritsch & Sambrook, *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning*, Vols. I and II (D. N. Glover ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); and the series *Methods in Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.).

Units, prefixes, and symbols may be denoted in their SI accepted form. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. Numeric ranges are inclusive of the numbers defining the range. Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes. The terms defined below are more fully defined by reference to the specification as a whole.



In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "microbe" is meant any microorganism (including both eukaryotic and prokaryotic microorganisms), such as fungi, yeast, bacteria, actinomycetes, algae and protozoa, as well as other unicellular structures.

A "fumonisin-producing microbe" is any microbe capable of producing the mycotoxin fumonisin or analogs thereof. Such microbes are generally members of the fungal genus *Fusarium*, as well as recombinantly derived organisms, which have been genetically altered to enable them to produce fumonisin or analogs thereof.

By "degrading fumonisin" is meant any modification to fumonisin or AP1 molecule which causes a decrease or loss in its toxic activity, such as degradation to less than 1%, 5%, 10%, or 50% of original toxicity, with less than 10% being preferred. Such a change can comprise cleavage of any of the various bonds, oxidation, reduction, the addition or deletion of a chemical moiety, or any other change that affects the activity of the molecule. In a preferred embodiment, the modification includes hydrolysis of the ester linkage in the molecule as a first step and then oxidative deamination. Furthermore, chemically altered fumonisin can be isolated from cultures of microbes that produce an enzyme of this invention, such as growing the organisms on media containing radioactively-labeled fumonisin, tracing the label, and isolating the degraded toxin for further study. The degraded fumonisin can be compared to the active compound for its phytotoxicity or mammalian toxicity in known sensitive species, such as porcines, rabbits, and equines or in cell or tissue culture assays. Such toxicity assays are known in the art. For example, in plants a whole leaf bioassay can be used in which solutions of the active and inactive compound are applied to the leaves of sensitive plants. The leaves may be treated *in situ* or, alternatively, excised leaves may be used. The relative toxicity of the compounds can be estimated by grading the ensuing damage to the plant tissues and by measuring the size of lesions formed within a given time period. Other known assays can be performed at the cellular level, employing standard tissue culture methodologies e.g., using cell suspension cultures.

By "fumonisin esterase" is meant any enzyme capable of hydrolysis of the ester linkage in fumonisin or a structurally similar molecule such as AAL toxin. Two examples of such enzymes are ESP1 and BEST1 found in US patent application no. 5,716,820,

issued February 10, 1998; US patent no. 5,792,931 issued August 11, 1998; and pending US application nos. 08/888,950 and 08/888,949, both filed July 7, 1997.

By "structurally related mycotoxin" is meant any mycotoxin having a chemical structure related to a fumonisin or AP1 such as AAL toxin, fumonisin B1, fumonisin B2, 5 fumonisin B3, fumonisin B4, fumonisin C1, fumonisin A1 and A2, and their analogs or hydrolyzed form, as well as other mycotoxins having similar chemical structures, including synthetically made analogs that contain a C-2 or C-1 amine group and one or more adjacent hydroxyl groups, that would be expected to be degraded by the activity of an enzyme of the present invention. The present invention is the first flavin amine oxidase 10 known to attack a primary amine not located at C-1 (i.e. C-2 of AP1) and resulting in a keto rather than an aldehydic product.

It is understood that "AP1" or "amino polyol" as used here is to designate the hydrolyzed form of any fumonisin, FB1, FB2, FB3, FB4, AAL, or any other AP1-like compound, including a compound made synthetically, that contains a C-2 or C-1 amine 15 group and one or more adjacent hydroxyl groups.

By "amplified" is meant the construction of multiple copies of a nucleic acid sequence or multiple copies complementary to the nucleic acid sequence using at least one of the nucleic acid sequences as a template. Amplification systems include the polymerase chain reaction (PCR) system, ligase chain reaction (LCR) system, nucleic acid sequence 20 based amplification (NASBA, Cangene, Mississauga, Ontario), Q-Beta Replicase systems, transcription-based amplification system (TAS), and strand displacement amplification (SDA). See, e.g., *Diagnostic Molecular Microbiology: Principles and Applications*, D. H. Persing *et al.*, Ed., American Society for Microbiology, Washington, DC (1993). The product of amplification is termed an amplicon.

25 The term "conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refer to those nucleic acids that encode identical or conservatively modified variants of the amino acid sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For 30 instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such

nucleic acid variations are "silent variations" and represent one species of conservatively modified variation. Every nucleic acid sequence herein that encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of ordinary skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only  
5 codon for methionine, one exception is *Micrococcus rubens*, for which GTG is the methionine codon (Ishizuka, *et al.*, *J. Gen'l Microbiol*, 139:425-432 (1993)) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid, which encodes a polypeptide of the present invention, is implicit in each described polypeptide sequence and incorporated herein by reference.

10 As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" when the alteration results in the substitution of an amino acid with a chemically similar amino acid. Thus,  
15 any number of amino acid residues selected from the group of integers consisting of from 1 to 15 can be so altered. Thus, for example, 1, 2, 3, 4, 5, 7, or 10 alterations can be made. Conservatively modified variants typically provide similar biological activity as the unmodified polypeptide sequence from which they are derived. For example, substrate specificity, enzyme activity, or ligand/receptor binding is generally at least 30%, 40%,  
20 50%, 60%, 70%, 80%, or 90%, preferably 60-90% of the native protein for it's native substrate. Conservative substitution tables providing functionally similar amino acids are well known in the art.

The following six groups each contain amino acids that are conservative substitutions for one another:

- 25 1) Alanine (A), Serine (S), Threonine (T);  
2) Aspartic acid (D), Glutamic acid (E);  
3) Asparagine (N), Glutamine (Q);  
4) Arginine (R), Lysine (K);  
5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and  
30 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

See also, Creighton (1984) *Proteins* W.H. Freeman and Company.

As used herein, "consisting essentially of" means the inclusion of additional sequences to an object polynucleotide where the additional sequences do not selectively hybridize, under stringent hybridization conditions, to the same cDNA as the polynucleotide and where the hybridization conditions include a wash step in 0.1X SSC and 0.1% sodium dodecyl sulfate at 65°C.

By "encoding" or "encoded", with respect to a specified nucleic acid, is meant comprising the information for translation into the specified protein. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid, or may lack such intervening non-translated sequences (e.g., as in cDNA). The information by which a protein is encoded is specified by the use of codons. Typically, the amino acid sequence is encoded by the nucleic acid using the "universal" genetic code. However, variants of the universal code, such as is present in some plant, animal, and fungal mitochondria, the bacterium *Mycoplasma capricolum* (*Proc. Natl. Acad. Sci. (USA)*, 82: 2306-2309 (1985)), or the ciliate *Macronucleus*, may be used when the nucleic acid is expressed using these organisms.

When the nucleic acid is prepared or altered synthetically, advantage can be taken of known codon preferences of the intended host where the nucleic acid is to be expressed. For example, although nucleic acid sequences of the present invention may be expressed in both monocotyledonous and dicotyledonous plant species, sequences can be modified to account for the specific codon preferences and GC content preferences of monocotyledonous plants or dicotyledonous plants as these preferences have been shown to differ (Murray *et al.* Nucl. Acids Res. 17: 477-498 (1989) and herein incorporated by reference). Thus, the maize preferred codon for a particular amino acid might be derived from known gene sequences from maize. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray *et al.*, *supra*.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous structural gene is from a species different from that from which the structural gene was derived, or, if from the same species, one or both are substantially modified from their original form. A

heterologous protein may originate from a foreign species or, if from the same species, is substantially modified from its original form by deliberate human intervention.

By "host cell" is meant a cell, which contains a vector and supports the replication and/or expression of the expression vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, plant, amphibian, or mammalian cells. Preferably, host cells are monocotyledonous or dicotyledonous plant cells, including but not limited to maize, sorghum, sunflower, soybean, wheat, alfalfa, rice, cotton, canola, barley, millet, and tomato. A particularly preferred monocotyledonous host cell is a maize host cell.

The term "hybridization complex" includes reference to a duplex nucleic acid structure formed by two single-stranded nucleic acid sequences selectively hybridized with each other.

The term "introduced" in the context of inserting a nucleic acid into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

The terms "isolated" refers to material, such as a nucleic acid or a protein, which is substantially or essentially free from components which normally accompany or interact with it as found in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment. Nucleic acids, which are "isolated", as defined herein, are also referred to as "heterologous" nucleic acids.

Unless otherwise stated, the term "APAO nucleic acid" means a nucleic acid comprising a polynucleotide ("APAO polynucleotide") encoding an APAO polypeptide. The term APAO, unless otherwise stated can encompass both APAO and the functional, truncated version of APAO designated trAPAO.

As used herein, "nucleic acid" includes reference to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues having the essential nature of natural nucleotides in that they hybridize to single-stranded nucleic acids in a manner similar to naturally occurring nucleotides (e.g., peptide nucleic acids).

By "nucleic acid library" is meant a collection of isolated DNA or RNA molecules, which comprise and substantially represent the entire transcribed fraction of a genome of a specified organism. Construction of exemplary nucleic acid libraries, such as genomic and cDNA libraries, is taught in standard molecular biology references such as Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology*, Vol. 152, Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, 2nd ed., Vol. 1-3 (1989); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, Eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994 Supplement).

As used herein "operably linked" includes reference to a functional linkage between a first sequence, such as a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

As used herein, the term "plant" includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds and plant cells and progeny of same. Plant cell, as used herein includes, without limitation, seeds suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants, which can be used in the methods of the invention, is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants including species from the genera: *Cucurbita*, *Rosa*, *Vitis*, *Juglans*, *Fragaria*, *Lotus*, *Medicago*, *Onobrychis*, *Trifolium*, *Trigonella*, *Vigna*, *Citrus*, *Linum*, *Geranium*, *Manihot*, *Daucus*, *Arabidopsis*, *Brassica*, *Raphanus*, *Sinapis*, *Atropa*, *Capsicum*, *Datura*, *Hyoscyamus*, *Lycopersicon*, *Nicotiana*, *Solanum*, *Petunia*, *Digitalis*, *Majorana*, *Cichorium*, *Helianthus*, *Lactuca*, *Bromus*, *Asparagus*, *Antirrhinum*, *Heterocallis*, *Nemesis*, *Pelargonium*, *Panicum*, *Pennisetum*, *Ranunculus*, *Senecio*, *Salpiglossis*, *Cucumis*, *Browaalia*, *Glycine*, *Pisum*, *Phaseolus*, *Lolium*, *Oryza*, *Avena*, *Hordeum*, *Secale*, *Allium*, and *Triticum*. A particularly preferred plant is *Zea mays*.

As used herein, "polynucleotide" includes reference to a deoxyribopolynucleotide, ribopolynucleotide, or analogs thereof that have the essential nature of a natural

ribonucleotide in that they hybridize, under stringent hybridization conditions, to substantially the same nucleotide sequence as naturally occurring nucleotides and/or allow translation into the same amino acid(s) as the naturally occurring nucleotide(s). A polynucleotide can be full-length or a subsequence of a native or heterologous structural or regulatory gene. Unless otherwise indicated, the term includes reference to the specified sequence as well as the complementary sequence thereof. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein. It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including *inter alia*, simple and complex cells.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

As used herein "promoter" includes reference to a region of DNA upstream from the start of transcription and involved in recognition and binding of RNA polymerase and other proteins to initiate transcription. A "plant promoter" is a promoter capable of initiating transcription in plant cells. Exemplary plant promoters include, but are not limited to, those that are obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells such *Agrobacterium* or *Rhizobium*. Examples are promoters that preferentially initiate transcription in certain tissues, such as leaves, roots, seeds, fibres, xylem vessels, tracheids, or sclerenchyma. Such promoters are referred to as "tissue preferred". A "cell type" specific promoter primarily drives expression in certain cell types in one or more organs, for example, vascular cells in roots or leaves. An "inducible" or "regulatable" promoter is a promoter, which is under environmental control. Examples of environmental conditions that may effect transcription by inducible promoters include anaerobic conditions or the presence of light. Another type of promoter is a developmentally regulated promoter, for example, a promoter that drives expression during

pollen development. Tissue preferred, cell type specific, developmentally regulated, and inducible promoters constitute the class of "non-constitutive" promoters. A "constitutive" promoter is a promoter, which is active under most environmental conditions.

The term "APAO polypeptide or trAPAO polypeptide" refers to one or more amino acid sequences. The term is also inclusive of fragments, variants, homologs, alleles or precursors (e.g., preproproteins or proproteins) thereof. An "APAO or trAPAO protein" comprises an APAO or trAPAO polypeptide.

As used herein "recombinant" includes reference to a cell or vector, that has been modified by the introduction of a heterologous nucleic acid or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found in identical form within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all as a result of deliberate human intervention. The term "recombinant" as used herein does not encompass the alteration of the cell or vector by naturally occurring events (e.g., spontaneous mutation, natural transformation/transduction/transposition) such as those occurring without deliberate human intervention.

As used herein, a "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements, which permit transcription of a particular nucleic acid in a target cell. The recombinant expression cassette can be incorporated into a plasmid, chromosome, mitochondrial DNA, plastid DNA, virus, or nucleic acid fragment. Typically, the recombinant expression cassette portion of an expression vector includes, among other sequences, a nucleic acid to be transcribed, and a promoter.

The term "residue" or "amino acid residue" or "amino acid" are used interchangeably herein to refer to an amino acid that is incorporated into a protein, polypeptide, or peptide (collectively "protein"). The amino acid may be a naturally occurring amino acid and, unless otherwise limited, may encompass known analogs of natural amino acids that can function in a similar manner as naturally occurring amino acids.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than



its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 40% sequence identity, preferably 60-90% sequence identity, and most preferably 100% sequence identity (i.e., complementary) with each other.

5       The terms “stringent conditions” or “stringent hybridization conditions” include reference to conditions under which a probe will hybridize to its target sequence, to a detectably greater degree than other sequences (e.g., at least 2-fold over background). Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing  
10       conditions, target sequences can be identified which can be up to 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Optimally, the probe is approximately 500 nucleotides in length, but can vary greatly in length from less than 500 nucleotides to equal to the entire length of  
15       the target sequence.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50  
20       nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide or Denhardt's. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include  
25       hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the  
30        $T_m$  can be approximated from the equation of Meinkoth and Wahl, *Anal. Biochem.*, 138:267-284 (1984):  $T_m = 81.5\text{ }^{\circ}\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$ ; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and

cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The  $T_m$  is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe.  $T_m$  is reduced by about 1 °C for each 1% of mismatching; thus,  $T_m$ , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with  $\geq 90\%$  identity are sought, the  $T_m$  can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point ( $T_m$ ) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point ( $T_m$ ); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point ( $T_m$ ); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point ( $T_m$ ). Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a  $T_m$  of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995). Unless otherwise stated, in the present application high stringency is defined as hybridization in 4X SSC, 5X Denhardt's (5g Ficoll, 5g polyvinylpyrrolidone, 5 g bovine serum albumin in 500ml of water), 0.1 mg/ml boiled salmon sperm DNA, and 25 mM Na phosphate at 65°C, and a wash in 0.1X SSC, 0.1% SDS at 65°C.

As used herein, "transgenic plant" includes reference to a plant, which comprises within its genome a heterologous polynucleotide. Generally, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated

into the genome alone or as part of a recombinant expression cassette. "Transgenic" is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

As used herein, "vector" includes reference to a nucleic acid used in transfection of a host cell and into which can be inserted a polynucleotide. Vectors are often replicons. Expression vectors permit transcription of a nucleic acid inserted therein.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides or polypeptides: (a) "reference sequence", (b) "comparison window", (c) "sequence identity", (d) "percentage of sequence identity", and (e) "substantial identity".

(a) As used herein, "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset or the entirety of a specified sequence; for example, as a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence.

(b) As used herein, "comparison window" means includes reference to a contiguous and specified segment of a polynucleotide sequence, wherein the polynucleotide sequence may be compared to a reference sequence and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Generally, the comparison window is at least 20 contiguous nucleotides in length, and optionally can be 30, 40, 50, 100, or longer. Those of skill in the art understand that to avoid a high similarity to a reference sequence due to inclusion of gaps in the polynucleotide sequence a gap penalty is typically introduced and is subtracted from the number of matches.

Methods of alignment of nucleotide and amino acid sequences for comparison are well known in the art. The local homology algorithm (Best Fit) of Smith and Waterman,

Adv. Appl. Math may conduct optimal alignment of sequences for comparison. 2: 482 (1981); by the homology alignment algorithm (GAP) of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970); by the search for similarity method (Tfasta and Fasta) of Pearson and Lipman, *Proc. Natl. Acad. Sci.* 85: 2444 (1988); by computerized implementations of these algorithms, including, but not limited to: CLUSTAL in the PC/Gene program by Intelligenetics, Mountain View, California, GAP, BESTFIT, BLAST, FASTA, and TFasta in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, Wisconsin, USA; the CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, *et al.*, *Nucleic Acids Research* 16: 10881-90 (1988); Huang, *et al.*, *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, *et al.*, *Methods in Molecular Biology* 24: 307-331 (1994). The preferred program to use for optimal global alignment of multiple sequences is PileUp (Feng and Doolittle, *Journal of Molecular Evolution*, 25:351-360 (1987) which is similar to the method described by Higgins and Sharp, *CABIOS*, 5:151-153 (1989) and hereby incorporated by reference). The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, *et al.*, Eds., Greene Publishing and Wiley-Interscience, New York (1995).

GAP uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48: 443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. GAP considers all possible alignments and gap positions and creates the alignment with the largest number of matched bases and the fewest gaps. It allows for the provision of a gap creation penalty and a gap extension penalty in units of matched bases. GAP must make a profit of gap creation penalty number of matches for each gap it inserts. If a gap extension penalty greater than zero is chosen, GAP must, in addition, make a profit for each gap inserted of the length of the gap times the gap extension penalty. Default gap creation penalty values and gap extension penalty

values in Version 10 of the Wisconsin Genetics Software Package are 8 and 2, respectively. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 100. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50, or greater.

GAP presents one member of the family of best alignments. There may be many members of this family, but no other member has a better quality. GAP displays four figures of merit for alignments: Quality, Ratio, Identity, and Similarity. The Quality is the metric maximized in order to align the sequences. Ratio is the quality divided by the number of bases in the shorter segment. Percent Identity is the percent of the symbols that actually match. Percent Similarity is the percent of the symbols that are similar. Symbols that are across from gaps are ignored. A similarity is scored when the scoring matrix value for a pair of symbols is greater than or equal to 0.50, the similarity threshold. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (see Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the BLAST 2.0 suite of programs using default parameters. Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402 (1997).

As those of ordinary skill in the art will understand, BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences, which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, *Comput. Chem.*, 17:149-163 (1993)) and XNU (Claverie and States, *Comput. Chem.*, 17:191-201 (1993)) low-complexity filters can be employed alone or in combination.

(c) As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences includes reference to the residues in the two sequences, which are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by

conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g. charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences, which differ by such conservative substitutions, are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to the algorithm of Meyers and Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988) e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, California, USA).

(d) As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

(e) (i) The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has between 50-100% sequence identity, preferably at least 50% sequence identity, preferably at least 60% sequence identity, preferably at least 70%, more preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using one of the alignment programs described using standard parameters. One of skill will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino

acid similarity, reading frame positioning and the like. Substantial identity of amino acid sequences for these purposes normally means sequence identity of between 55-100%, preferably at least 55%, preferably at least 60%, more preferably at least 70%, 80%, 90%, and most preferably at least 95%.

5 Another indication that nucleotide sequences are substantially identical is if two molecules hybridize to each other under stringent conditions. The degeneracy of the genetic code allows for many amino acids substitutions that lead to variety in the nucleotide sequence that code for the same amino acid, hence it is possible that the DNA sequence could code for the same polypeptide but not hybridize to each other under  
10 stringent conditions. This may occur, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. One indication that two nucleic acid sequences are substantially identical is that the polypeptide, which the first nucleic acid encodes, is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

15 (e) (ii) The terms "substantial identity" in the context of a peptide indicates that a peptide comprises a sequence with between 55-100% sequence identity to a reference sequence preferably at least 55% sequence identity, preferably 60% preferably 70%, more preferably 80%, most preferably at least 90% or 95% sequence identity to the reference sequence over a specified comparison window. Preferably, optimal alignment is  
20 conducted using the homology alignment algorithm of Needleman and Wunsch, *J. Mol. Biol.* 48: 443 (1970). An indication that two peptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the second peptide. Thus, a peptide is substantially identical to a second peptide, for example, where the two peptides differ only by a conservative substitution. In addition, a peptide can be  
25 substantially identical to a second peptide when they differ by a non-conservative change if the epitope that the antibody recognizes is substantially identical. Peptides, which are "substantially similar" share sequences as, noted above except that residue positions, which are not identical, may differ by conservative amino acid changes.

### 30 **Fumonisin Degrading Organisms**

The present invention is based on the discovery of organisms with the ability to degrade the mycotoxin fumonisin. In a search for a biological means of detoxifying

fumonisin, several dematiaceous hyphomycetes were isolated from field-grown maize kernels. The fungi were found to be capable of growing on fumonisin B1 or B2 (FB1 or FB2) as a sole carbon source, degrading it partially or completely in the process. One species, identified as *Exophiala spinifera*, a "black yeast", was recovered from maize seed  
5 from diverse locations in the southeastern and south central United States. The enzyme-active strain of *Exophiala spinifera* (ATCC 74269) was deposited (see US patent application no. 5,716,820, issued February 10, 1998; US patent no. 5,792,931 issued August 11, 1998; and pending US application nos. 08/888,950 and 08/888,949, both filed July 7, 1997).

## **Nucleic Acids**

The present invention provides, *inter alia*, isolated nucleic acids of RNA, DNA, and analogs and/or chimeras thereof, comprising an APAO or trAPAO polynucleotide.

The present invention also includes polynucleotides optimized for expression in  
15 different organisms. For example, for expression of the polynucleotide in a maize plant, the sequence can be altered to account for specific codon preferences and to alter GC content as according to Murray *et al*, *supra*. Maize codon usage for 28 genes from maize plants is listed in Table 4 of Murray, *et al*, *supra*.

The APAO or trAPAO nucleic acids of the present invention comprise isolated  
20 APAO or trAPAO polynucleotides which, are inclusive of:

(a) a polynucleotide encoding an APAO or trAPAO polypeptide of the sequences found in SEQ ID NO: 6 and 22, and conservatively modified and polymorphic variants thereof;

(b) a polynucleotide which selectively hybridizes to a polynucleotide of (a) or (b);

25 (c) a polynucleotide having at least 40% sequence identity with polynucleotides of (a) or (b);

(d) complementary sequences of polynucleotides of (a), (b), or (c); and

(e) a polynucleotide comprising at least 15 contiguous nucleotides from a polynucleotide of (a), (b), (c), or (d).

30 In addition, polynucleotides are presented that are a fusion of an APAO or trAPAO polynucleotide and the polynucleotide of a fumonisin esterase. The invention encompasses the sequences from *Exophiala* as well as sequences having sequence



similarity with such sequences. It is recognized that the sequences of the invention can be used to isolate corresponding sequences in other organisms. Methods such as PCR, hybridization, and the like can be used to identify sequences having substantial sequence similarity to the sequences of the invention. See, for example, Sambrook, *et al.*, (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Planview, New York) and Innis *et al.*, (1990) *PCR Protocols: Guide to Methods and Applications* (Academic Press, New York). Coding sequences isolated based on their sequence identity to the entire fumonisin degrading coding sequences set forth herein or to fragments thereof are encompassed by the present invention.

It is recognized that the sequences of the invention can be used to isolate similar sequences from other fumonisin degrading organisms. Likewise sequences from other fumonisin degrading organisms may be used in combination with the sequences of the present invention. See, for example, copending application entitled "Compositions and Methods for Fumonisin Detoxification", U.S. application serial number 60/092,953, filed concurrently herewith and herein incorporated by reference.

Plasmids containing the polynucleotide sequences of the invention were deposited with American Type Culture Collection (ATCC), Manassas, Virginia, and assigned Accession Nos. 98812, 98813, 98814, 98815, and 98816. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. § 112.

### **Construction of Nucleic Acids**

The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, or combinations thereof. In some embodiments, the polynucleotides of the present invention will be cloned, amplified, or otherwise constructed from a fungus or bacteria.

The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences may be inserted to aid in the

isolation of the translated polynucleotide of the present invention. For example, a hexahistidine marker sequence provides a convenient means to purify the proteins of the present invention. The nucleic acid of the present invention - excluding the polynucleotide sequence - is optionally a vector, adapter, or linker for cloning and/or expression of a polynucleotide of the present invention. Additional sequences may be added to such cloning and/or expression sequences to optimize their function in cloning and/or expression, to aid in isolation of the polynucleotide, or to improve the introduction of the polynucleotide into a cell. Typically, the length of a nucleic acid of the present invention less the length of its polynucleotide of the present invention is less than 20 kilobase pairs, often less than 15 kb, and frequently less than 10 kb. Use of cloning vectors, expression vectors, adapters, and linkers is well known in the art. Exemplary nucleic acids include such vectors as: M13, lambda ZAP Express, lambda ZAP II, lambda gt10, lambda gt11, pBK-CMV, pBK-RSV, pBluescript II, lambda DASH II, lambda EMBL 3, lambda EMBL 4, pWE15, SuperCos 1, SurfZap, Uni-ZAP, pBC, pBS+/-, pSG5, pBK, pCR-Script, pET, pSPUTK, p3'SS, pGEM, pSK+/-, pGEX, pSPORTI and II, pOPRSVI CAT, pOPI3 CAT, pXT1, pSG5, pPbac, pMbac, pMC1neo, pOG44, pOG45, pFRT $\beta$ GAL, pNEO $\beta$ GAL, pRS403, pRS404, pRS405, pRS406, pRS413, pRS414, pRS415, pRS416, lambda MOSSlox, and lambda MOSElox. Optional vectors for the present invention, include but are not limited to, lambda ZAP II, and pGEX. For a description of various nucleic acids see, for example, Stratagene Cloning Systems, Catalogs 1995, 1996, 1997 (La Jolla, CA); and, Amersham Life Sciences, Inc, Catalog '97 (Arlington Heights, IL).

### Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68: 90-99 (1979); the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68: 109-151 (1979); the diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.* 22: 1859-1862 (1981); the solid phase phosphoramidite triester method described by Beaucage and Caruthers, *Tetra. Letts.* 22(20): 1859-1862 (1981), *e.g.*, using an automated synthesizer, *e.g.*, as described in Needham-VanDevanter *et al.*, *Nucleic Acids Res.*, 12: 6159-6168 (1984); and, the solid support method of US Patent No. 4,458,066. Chemical

synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100  
5 bases, longer sequences may be obtained by the ligation of shorter sequences.

### UTRs and Codon Preference

In general, translational efficiency has been found to be regulated by specific sequence elements in the 5' non-coding or untranslated region (5' UTR) of the RNA.  
10 Positive sequence motifs include translational initiation consensus sequences (Kozak, *Nucleic Acids Res.* 15:8125 (1987)) and the 5' <G> 7 methyl GpppG RNA cap structure (Drummond *et al.*, *Nucleic Acids Res.* 13:7375 (1985)). Negative elements include stable intramolecular 5' UTR stem-loop structures (Muesing *et al.*, *Cell* 48:691 (1987)) and AUG sequences or short open reading frames preceded by an appropriate AUG in the 5' UTR  
15 (Kozak, *supra*, Rao *et al.*, *Mol. and Cell. Biol.* 8:284 (1988)). Accordingly, the present invention provides 5' and/or 3' UTR regions for modulation of translation of heterologous coding sequences.

Further, the polypeptide-encoding segments of the polynucleotides of the present invention can be modified to alter codon usage. Altered codon usage can be employed to  
20 alter translational efficiency and/or to optimize the coding sequence for expression in a desired host or to optimize the codon usage in a heterologous sequence for expression in maize. Codon usage in the coding regions of the polynucleotides of the present invention can be analyzed statistically using commercially available software packages such as "Codon Preference" available from the University of Wisconsin Genetics Computer Group  
25 (see Devereaux *et al.*, *Nucleic Acids Res.* 12: 387-395 (1984)) or MacVector 4.1 (Eastman Kodak Co., New Haven, Conn.). Thus, the present invention provides a codon usage frequency characteristic of the coding region of at least one of the polynucleotides of the present invention. The number of polynucleotides (3 nucleotides per amino acid) that can be used to determine a codon usage frequency can be any integer from 3 to the number of  
30 polynucleotides of the present invention as provided herein. Optionally, the polynucleotides will be full-length sequences. An exemplary number of sequences for statistical analysis can be at least 1, 5, 10, 20, 50, or 100.

## Sequence Shuffling

The present invention provides methods for sequence shuffling using polynucleotides of the present invention, and compositions resulting therefrom. Sequence shuffling is described in PCT publication No. 96/19256. See also, Zhang, J.- H., *et al.* 5 *Proc. Natl. Acad. Sci. USA* 94:4504-4509 (1997) and Zhao, *et al.*, *Nature Biotech* 16:258-261 (1998). Generally, sequence shuffling provides a means for generating libraries of polynucleotides having a desired characteristic, which can be selected or screened for. Libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides, which comprise sequence regions, which have substantial 10 sequence identity and can be homologously recombined in vitro or in vivo. The population of sequence-recombined polynucleotides comprises a subpopulation of polynucleotides which possess desired or advantageous characteristics and which can be selected by a suitable selection or screening method. The characteristics can be any property or attribute capable of being selected for or detected in a screening system, and 15 may include properties of: an encoded protein, a transcriptional element, a sequence controlling transcription, RNA processing, RNA stability, chromatin conformation, translation, or other expression property of a gene or transgene, a replicative element, a protein-binding element, or the like, such as any feature which confers a selectable or detectable property. In some embodiments, the selected characteristic will be an altered 20  $K_m$  and/or  $K_{cat}$  over the wild-type protein as provided herein. In other embodiments, a protein or polynucleotide generated from sequence shuffling will have a ligand binding affinity greater than the non-shuffled wild-type polynucleotide. In yet other embodiments, a protein or polynucleotide generated from sequence shuffling will have an altered pH optimum as compared to the non-shuffled wild-type polynucleotide. The increase in such 25 properties can be at least 110%, 120%, 130%, 140% or greater than 150% of the wild-type value.

## Recombinant Expression Cassettes

The present invention further provides recombinant expression cassettes 30 comprising a nucleic acid of the present invention. A nucleic acid sequence coding for the desired polynucleotide of the present invention, for example a cDNA or a genomic sequence encoding a polypeptide long enough to code for an active protein of the present

invention, can be used to construct a recombinant expression cassette which can be introduced into the desired host cell. A recombinant expression cassette will typically comprise a polynucleotide of the present invention operably linked to transcriptional initiation regulatory sequences which will direct the transcription of the polynucleotide in the intended host cell, such as tissues of a transformed plant.

For example, plant expression vectors may include (1) a cloned plant gene under the transcriptional control of 5' and 3' regulatory sequences and (2) a dominant selectable marker. Such plant expression vectors may also contain, if desired, a promoter regulatory region (e.g., one conferring inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific/selective expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

A plant promoter fragment can be employed which will direct expression of a polynucleotide of the present invention in all tissues of a regenerated plant. Such promoters are referred to herein as "constitutive" promoters and are active under most environmental conditions and states of development or cell differentiation. Examples of constitutive promoters include the 1'- or 2'- promoter derived from T-DNA of *Agrobacterium tumefaciens*, the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US Patent No. 5,683,439), the *Nos* promoter, the rubisco promoter, the GRP1-8 promoter, the 35S promoter from cauliflower mosaic virus (CaMV), as described in Odell et al., (1985), *Nature*, 313:810-812, rice actin (McElroy et al., (1990), *Plant Cell*, 163-171); ubiquitin (Christensen et al., (1992), *Plant Mol. Biol.* 12:619-632; and Christensen, et al., (1992), *Plant Mol. Biol.* 18:675-689); pEMU (Last, et al., (1991), *Theor. Appl. Genet.* 81:581-588); MAS (Velten et al., (1984), *EMBO J.* 3:2723-2730); and maize H3 histone (Lepetit et al., (1992), *Mol. Gen. Genet.* 231:276-285; and Atanassova et al., (1992), *Plant Journal* 2(3):291-300), ALS promoter, as described in published PCT Application WO 96/30530, and other transcription initiation regions from various plant genes known to those of skill. For the present invention ubiquitin is the preferred promoter for expression in monocot plants.

Alternatively, the plant promoter can direct expression of a polynucleotide of the present invention in a specific tissue or may be otherwise under more precise environmental or developmental control. Such promoters are referred to here as

"inducible" promoters. Environmental conditions that may effect transcription by inducible promoters include pathogen attack, anaerobic conditions, or the presence of light. Examples of inducible promoters are the Adh1 promoter, which is inducible by hypoxia or cold stress, the Hsp70 promoter, which is inducible by heat stress, and the PPDK promoter, which is inducible by light.

Examples of promoters under developmental control include promoters that initiate transcription only, or preferentially, in certain tissues, such as leaves, roots, fruit, seeds, or flowers. The operation of a promoter may also vary depending on its location in the genome. Thus, an inducible promoter may become fully or partially constitutive in certain locations.

If polypeptide expression is desired, it is generally desirable to include a polyadenylation region at the 3'-end of a polynucleotide coding region. The polyadenylation region can be derived from a variety of plant genes, or from T-DNA. The 3' end sequence to be added can be derived from, for example, the nopaline synthase or octopine synthase genes, or alternatively from another plant gene, or less preferably from any other eukaryotic gene. Examples of such regulatory elements include, but are not limited to, 3' termination and/or polyadenylation regions such as those of the *Agrobacterium tumefaciens* nopaline synthase (nos) gene (Bevan et al., (1983), *Nucl. Acids Res.* 12:369-385); the potato proteinase inhibitor II (PINII) gene (Keil, et al., (1986), *Nucl. Acids Res.* 14:5641-5650; and An et al., (1989), *Plant Cell* 1:115-122); and the CaMV 19S gene (Mogen et al., (1990), *Plant Cell* 2:1261-1272).

An intron sequence can be added to the 5' untranslated region or the coding sequence of the partial coding sequence to increase the amount of the mature message that accumulates in the cytosol. Inclusion of a spliceable intron in the transcription unit in both plant and animal expression constructs has been shown to increase gene expression at both the mRNA and protein levels up to 1000-fold. Buchman and Berg, *Mol. Cell Biol.* 8: 4395-4405 (1988); Callis et al., *Genes Dev.* 1: 1183-1200 (1987). Such intron enhancement of gene expression is typically greatest when placed near the 5' end of the transcription unit. Use of maize introns Adh1-S intron 1, 2, and 6, the Bronze-1 intron are known in the art. See generally, *The Maize Handbook*, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994).

Plant signal sequences, including, but not limited to, signal-peptide encoding DNA/RNA sequences which target proteins to the extracellular matrix of the plant cell (Dratewka-Kos, et al., (1989), *J. Biol. Chem.* 264:4896-4900), the *Nicotiana plumbaginifolia* extension gene (DeLoose, et al., (1991), *Gene* 99:95-100), signal peptides which target proteins to the vacuole like the sweet potato sporamin gene (Matsuka, et al., (1991), *PNAS* 88:834) and the barley lectin gene (Wilkins, et al., (1990), *Plant Cell*, 2:301-313), signal peptides which cause proteins to be secreted such as that of PR1b (Lind, et al., (1992), *Plant Mol. Biol.* 18:47-53), or the barley alpha amylase (BAA) (Rahmatullah, et al., *Plant Mol. Biol.* 12:119 (1989)) and hereby incorporated by reference), or from the present invention the signal peptide from the ESP1 or BEST1 gene, or signal peptides which target proteins to the plastids such as that of rapeseed enoyl-Acp reductase (Verwaert, et al., (1994), *Plant Mol. Biol.* 26:189-202) are useful in the invention. The barley alpha amylase signal sequence fused to the trAPAO polynucleotide (see SEQ ID NO: 20) is the preferred construct for expression in maize for the present invention.

The vector comprising the sequences from a polynucleotide of the present invention will typically comprise a marker gene, which confers a selectable phenotype on plant cells. Usually, the selectable marker gene will encode antibiotic resistance, with suitable genes including genes coding for resistance to the antibiotic spectinomycin (e.g., the aada gene), the streptomycin phosphotransferase (SPT) gene coding for streptomycin resistance, the neomycin phosphotransferase (NPTII) gene encoding kanamycin or geneticin resistance, the hygromycin phosphotransferase (HPT) gene coding for hygromycin resistance, genes coding for resistance to herbicides which act to inhibit the action of acetolactate synthase (ALS), in particular the sulfonylurea-type herbicides (e.g., the acetolactate synthase (ALS) gene containing mutations leading to such resistance in particular the S4 and/or Hra mutations), genes coding for resistance to herbicides which act to inhibit action of glutamine synthase, such as phosphinothricin or basta (e.g., the *bar* gene), or other such genes known in the art. The *bar* gene encodes resistance to the herbicide basta, and the ALS gene encodes resistance to the herbicide chlorsulfuron.

Alternatively, the invention, itself, could be used as a method for selection of transformants, in other words as a selectable marker. An APAAO or trAPAAO polynucleotide operably linked to a promoter and then transformed into a plant cell by any of the methods described in the present application would express the degradative enzyme.

When the plant cells are placed in the presence of either AP1 or a phytotoxic analog in culture only the transformed cells would be able to grow. In another embodiment, the plant cell could be transformed with both a polynucleotide for APAO and a polynucleotide for fumonisin esterase. The selective agent in this case could be either AP1 or fumonisin or any structural analog. Thus, growth of plant cells in the presence of a mycotoxin favors the survival of plant cells that have been transformed to express the coding sequence that codes for one of the enzymes of this invention and degrades the toxin. When the APAO or trAPAO cassette with or without the fumonisin esterase polynucleotide, is co-transformed with another gene of interest and then placed in the presence of fumonisin, AP1 or a phytotoxic analog, this invention would allow for selection of only those plant cells that contain the gene of interest. In the past antibiotic resistance genes have been used as selectable markers. Given the current concerns by consumers and environmentalist over use of antibiotic genes and the possibility of resistant microorganisms arising due to this use, a non-antibiotic resistant selectable marker system such as the present invention, fulfills this very important need.

Typical vectors useful for expression of genes in higher plants are well known in the art and include vectors derived from the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* described by Rogers *et al.*, Meth. In Enzymol., 153:253-277 (1987). These vectors are plant integrating vectors in that on transformation, the vectors integrate a portion of vector DNA into the genome of the host plant. Exemplary *A. tumefaciens* vectors useful herein are plasmids pKYLX6 and pKYLX7 of Schardl *et al.*, Gene, 61:1-11 (1987) and Berger *et al.*, Proc. Natl. Acad. Sci. U.S.A., 86:8402-8406 (1989). Another useful vector herein is plasmid pBI101.2 that is available from CLONTECH Laboratories, Inc. (Palo Alto, CA).

### Expression of Proteins in Host Cells

Using the nucleic acids of the present invention, one may express a protein of the present invention in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. The cells produce the protein in a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.



It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

5 In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and  
10 translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter, such as ubiquitin, to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Constitutive promoters are classified as providing for a range of constitutive expression.  
15 Thus, some are weak constitutive promoters, and others are strong constitutive promoters. Generally, by “weak promoter” is intended a promoter that drives expression of a coding sequence at a low level. By “low level” is intended at levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a “strong promoter” drives expression of a coding sequence at a “high level”, or about 1/10  
20 transcripts to about 1/100 transcripts to about 1/1,000 transcripts.

One of skill would recognize that modifications could be made to a protein of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a  
25 fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (*e.g.*, poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

#### 30 *A. Expression in Prokaryotes*

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli*; however, other microbial strains may also be

used. Commonly used prokaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.*, Nature 292:128 (1981)). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction of the gene of interest into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, *Gene* 22: 229-235 (1983); Mosbach, *et al.*, *Nature* 302: 543-545 (1983)). The pGEX-4T-1 plasmid vector from Pharmacia is the preferred *E. coli* expression vector for the present invention.

#### *B. Expression in Eukaryotes*

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. Sherman, F., *et al.*, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory (1982) is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeasts for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression control sequences, such as promoters, including

3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates or the pellets. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.*, *Immunol. Rev.* 89: 49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992).

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth, and *Drosophila* cell lines such as a Schneider cell line (See Schneider, *J. Embryol. Exp. Morphol.* 27: 353-365 (1987)).

As with yeast, when higher animal or plant host cells are employed, polyadenylation or transcription terminator sequences are typically incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, *J. Virol.* 45: 773-781 (1983)). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., Bovine Papilloma Virus DNA a Eukaryotic

Cloning Vector in *DNA Cloning Vol. II a Practical Approach*, D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238 (1985).

In addition, one of the genes for fumonisin esterase or the APAO or trAPAO placed in the appropriate plant expression vector can be used to transform plant cells. The enzyme can then be isolated from plant callus or the transformed cells can be used to regenerate transgenic plants. Such transgenic plants can be harvested, and the appropriate tissues (seed or leaves, for example) can be subjected to large scale protein extraction and purification techniques, and the fumonisin degradation enzymes or APAO can be isolated for use in fumonisin and fumonisin hydrolysis product detoxification processes.

### Plant Transformation Methods

Numerous methods for introducing foreign genes into plants are known and can be used to insert an APAO or trAPAO polynucleotide into a plant host, including biological and physical plant transformation protocols. See, for example, Miki et al., (1993), "Procedure for Introducing Foreign DNA into Plants", In: *Methods in Plant Molecular Biology and Biotechnology*, Glick and Thompson, eds., CRC Press, Inc., Boca Raton, pages 67-88. The methods chosen vary with the host plant, and include chemical transfection methods such as calcium phosphate, microorganism-mediated gene transfer such as *Agrobacterium* (Horsch, et al., (1985), *Science* 227:1229-31), electroporation, micro-injection, and biolistic bombardment.

Expression cassettes and vectors and *in vitro* culture methods for plant cell or tissue transformation and regeneration of plants are known and available. See, for example, Gruber, et al., (1993), "Vectors for Plant Transformation" In: *Methods in Plant Molecular Biology and Biotechnology*, Glick and Thompson, eds. CRC Press, Inc., Boca Raton, pages 89-119.

### *Agrobacterium*-mediated Transformation

The most widely utilized method for introducing an expression vector into plants is based on the natural transformation system of *Agrobacterium*. *A. tumefaciens* and *A. rhizogenes* are plant pathogenic soil bacteria, which genetically transform plant cells. The Ti and Ri plasmids of *A. tumefaciens* and *A. rhizogenes*, respectively, carry genes responsible for genetic transformation of plants. See, for example, Kado, (1991), *Crit.*

Rev. Plant Sci. 10:1. Descriptions of the *Agrobacterium* vector systems and methods for *Agrobacterium*-mediated gene transfer are provided in Gruber et al., *supra*; Miki, et al., *supra*; and Moloney et al., (1989), *Plant Cell Reports* 8:238.

Similarly, the gene can be inserted into the T-DNA region of a Ti or Ri plasmid  
5 derived from *A. tumefaciens* or *A. rhizogenes*, respectively. Thus, expression cassettes can  
be constructed as above, using these plasmids. Many control sequences are known which  
when coupled to a heterologous coding sequence and transformed into a host organism  
show fidelity in gene expression with respect to tissue/organ specificity of the original  
coding sequence. See, e.g., Benfey, P. N., and Chua, N. H. (1989) *Science* 244: 174-181.  
10 Particularly suitable control sequences for use in these plasmids are promoters for  
constitutive leaf-specific expression of the gene in the various target plants. Other useful  
control sequences include a promoter and terminator from the nopaline synthase gene  
(NOS). The NOS promoter and terminator are present in the plasmid pARC2, available  
from the American Type Culture Collection and designated ATCC 67238. If such a  
15 system is used, the virulence (*vir*) gene from either the Ti or Ri plasmid must also be  
present, either along with the T-DNA portion, or via a binary system where the *vir* gene is  
present on a separate vector. Such systems, vectors for use therein, and methods of  
transforming plant cells are described in US Pat. No. 4,658,082; US application Ser. No.  
913,914, filed Oct. 1, 1986, as referenced in US Patent 5,262,306, issued November 16,  
20 1993 to Robeson, et al.; and Simpson, R. B., et al. (1986) *Plant Mol. Biol.* 6: 403-415 (also  
referenced in the '306 patent); all incorporated by reference in their entirety.

Once constructed, these plasmids can be placed into *A. rhizogenes* or *A. tumefaciens*  
and these vectors used to transform cells of plant species, which are ordinarily  
susceptible to *Fusarium* or *Alternaria* infection. Several other transgenic plants are also  
25 contemplated by the present invention including but not limited to soybean, corn, sorghum,  
alfalfa, rice, clover, cabbage, banana, coffee, celery, tobacco, cowpea, cotton, melon and  
pepper. The selection of either *A. tumefaciens* or *A. rhizogenes* will depend on the plant  
being transformed thereby. In general *A. tumefaciens* is the preferred organism for  
transformation. Most dicotyledonous plants, some gymnosperms, and a few  
30 monocotyledonous plants (e.g. certain members of the *Liliales* and *Arales*) are susceptible  
to infection with *A. tumefaciens*. *A. rhizogenes* also has a wide host range, embracing most  
dicots and some gymnosperms, which includes members of the *Leguminosae*, *Compositae*,

and *Chenopodiaceae*. Monocot plants can now be transformed with some success. European Patent Application Publication Number 604 662 A1 to Hiei *et al.* discloses a method for transforming monocots using *Agrobacterium*. Saito *et al.* discloses a method for transforming monocots with *Agrobacterium* using the scutellum of immature embryos  
5 (European Application 672 752 A1). Ishida *et al.* discusses a method for transforming maize by exposing immature embryos to *A. tumefaciens* (Ishida *et al.*, *Nature Biotechnology*, 1996, 14:745-750).

Once transformed, these cells can be used to regenerate transgenic plants, capable of degrading fumonisin. For example, whole plants can be infected with these vectors by  
10 wounding the plant and then introducing the vector into the wound site. Any part of the plant can be wounded, including leaves, stems and roots. Alternatively, plant tissue, in the form of an explant, such as cotyledonary tissue or leaf disks, can be inoculated with these vectors, and cultured under conditions, which promote plant regeneration. Roots or shoots transformed by inoculation of plant tissue with *A. rhizogenes* or *A. tumefaciens*, containing  
15 the gene coding for the fumonisin degradation enzyme, can be used as a source of plant tissue to regenerate fumonisin-resistant transgenic plants, either via somatic embryogenesis or organogenesis. Examples of such methods for regenerating plant tissue are disclosed in Shahin, E. A. (1985) *Theor. Appl. Genet.* 69:235-240; US Pat. No. 4,658,082; Simpson, R. B., et al. (1986) *Plant Mol. Biol.* 6: 403-415; and U.S. patent applications Ser. Nos.  
20 913,913 and 913,914, both filed Oct. 1, 1986, as referenced in U.S. Patent 5,262,306, issued November 16, 1993 to Robeson, et al.; the entire disclosures therein incorporated herein by reference.

### **Direct Gene Transfer**

25 Despite the fact that the host range for *Agrobacterium*-mediated transformation is broad, some major cereal crop species and gymnosperms have generally been recalcitrant to this mode of gene transfer, even though some success has recently been achieved in rice (Hiei *et al.*, (1994), *The Plant Journal* 6:271-282). Several methods of plant transformation, collectively referred to as direct gene transfer, have been developed as an  
30 alternative to *Agrobacterium*-mediated transformation.

A generally applicable method of plant transformation is microprojectile-mediated transformation, where DNA is carried on the surface of microprojectiles measuring about 1

to 4  $\mu\text{m}$ . The expression vector is introduced into plant tissues with a biolistic device that accelerates the microprojectiles to speeds of 300 to 600 m/s which is sufficient to penetrate the plant cell walls and membranes. (Sanford et al., (1987), *Part. Sci. Technol.* 5:27; Sanford, 1988, *Trends Biotech* 6:299; Sanford, (1990), *Physiol. Plant* 79:206; Klein et al.,  
5 (1992), *Biotechnology* 10:268).

Another method for physical delivery of DNA to plants is sonication of target cells as described in Zang et al., (1991), *BioTechnology* 9:996. Alternatively, liposome or spheroplast fusions have been used to introduce expression vectors into plants. See, for example, Deshayes et al., (1985), *EMBO J.* 4:2731; and Christou et al., (1987), *PNAS USA*  
10 84:3962. Direct uptake of DNA into protoplasts using  $\text{CaCl}_2$  precipitation, polyvinyl alcohol, or poly-L-ornithine has also been reported. See, for example, Hain et al., (1985), *Mol. Gen. Genet.* 199:161; and Draper et al., (1982), *Plant Cell Physiol.* 23:451.

Electroporation of protoplasts and whole cells and tissues has also been described. See, for example, Donn et al., (1990), In: *Abstracts of the VIIth Int'l. Congress on Plant*  
15 *Cell and Tissue Culture IAPTC*, A2-38, page 53; D'Halluin et al., (1992), *Plant Cell* 4:1495-1505; and Spencer et al., (1994), *Plant Mol. Biol.* 24:51-61.

Thus, polynucleotide encoding a polypeptide able to inactivate fumonisin or AP1 can be isolated and cloned in an appropriate vector and inserted into an organism normally sensitive to the *Fusarium* or its toxins. Furthermore, the polynucleotide imparting  
20 fumonisin or AP1 degradative activity can be transferred into a suitable plasmid, and transformed into a plant. Thus, a fumonisin or AP1 degrading transgenic plant can be produced. Organisms expressing the polynucleotide can be easily identified by their ability to degrade fumonisin or AP1. The protein capable of degrading fumonisin or AP1 can be isolated and characterized using techniques well known in the art.

25

#### **APAO or trAPAO in a Transgenic Plant**

Fumonisin esterase reduces but does not eliminate the toxicity of fumonisins. Therefore a second enzymatic modification to further reduce or abolish toxicity is desirable. The partially purified APAO enzyme from *Exophiala spinifera* has little or no  
30 activity on intact FB1, a form of fumonisin. However, recombinant APAO enzyme from *Exophiala spinifera*, expressed in *E. coli*, has significant but reduced activity on intact FB1 and other B-series fumonisins. APAO or trAPAO thus could potentially be used without

fumonisin esterase since the amine group is the major target for detoxification. Alternatively, the two genes, fumonisin esterase and APAO (or trAPAO) can be used together for degrading toxins.

APAO is predicted to be an enzyme that, when by itself or co-expressed in a heterologous expression system along with fumonisin esterase (either ESP1 or BEST1), will result in the production of 2-oxo pentol (2-OP) from fumonisin B1. The substrate range of recombinant, *E. coli*-expressed APAO is limited to fumonisins and their hydrolysis products and does not include amino acids, sphingolipid precursors such as phytosphingosine, or polyamines such as spermidine. Thus, APAO is highly specific for fumonisin-like amines, and thus would have little deleterious effect on other cellular metabolites. In addition, if it is extracellularly localized, it will limit any contact with biologically important amines that might also be substrates. The end result will be a more effective detoxification of fumonisins than can be achieved with esterase alone.

The oxidase activity of APAO is predicted to result in generation of hydrogen peroxide in stoichiometric amounts relative to AP1 or fumonisin oxidized. This may prove to be an additional benefit of this enzyme, since hydrogen peroxide is both antimicrobial and is thought to contribute to the onset of a defense response in plants (Przemylaw, *Biochem J.*, 322:681-692 (1997), Lamb, *et al.*, *Ann Rev Plant Physiol Plant Mol Bio* 48:251-275 (1997), and Alvarez, *et al.*, *Oxidative Stress and the Molecular Biology of Antioxidant Defenses*, Cold Spring Harbor Press, 815-839 (1997)).

Since one of the preferred embodiments of the present invention is to have both a fumonisin esterase polynucleotide and an APAO or trAPAO polynucleotide present in a plant, there are several ways to introduce more than one polynucleotide in a plant. One way is to transform plant tissue with polynucleotides to both fumonisin esterase and APAO or trAPAO at the same time. In some tissue culture systems it is possible to transform callus with one polynucleotide and then after establishing a stable culture line containing the first polynucleotide, transform the callus a second time with the second polynucleotide. One could also transform plant tissue with one polynucleotide, regenerate whole plants, then transform the second polynucleotide into plant tissue and regenerate whole plants. The final step would then be to cross a plant containing the first polynucleotide with a plant containing the second polynucleotide and select for progeny containing both polynucleotides.



Another method is to create a fusion protein between esterase and APAO or trAPAO, preferably with a spacer region between the two polypeptides. Both enzymes would be active although tethered to each other. In addition, an enzyme cleavage site engineered in the spacer region, would allow cleavage by an endogenous or introduced protease.

5 Transgenic plants containing both a fumonisin esterase enzyme and/or the APAO enzyme and thus able to degrade fumonisin or a structurally related mycotoxin would be able to reduce or eliminate the pathogenicity of any microorganism that uses fumonisin or a structurally related mycotoxin as a mode of entry to infect a plant. Fungal pathogens frequently use toxins to damage plants and weaken cell integrity in order to gain entry and  
10 expand infection in a plant. By preventing the damage induced by a toxin, a plant would be able to prevent the establishment of the pathogen and thereby become tolerant or resistant to the pathogen.

Another benefit of fumonisin degradation is the production of hydrogen peroxide. When fumonisin is broken down to 2-OP, hydrogen peroxide is produced as a by-product.  
15 Hydrogen peroxide production can trigger enhanced resistance responses in a number of ways. 1) Hydrogen peroxide has direct antimicrobial activity. 2) Hydrogen peroxide acts as a substrate for peroxidases associated with lignin polymerization and hence cell wall strengthening. 3) Via still to be determined mechanisms, hydrogen peroxide acts as a signal for activation of expression of defense related genes, including those that result in  
20 stimulation of salicylic acid accumulation. Salicylic acid is thought to act an endogenous signal molecule that triggers expression of genes coding for several classes of pathogenesis-related proteins. Moreover, salicylic acid may set up the oxidative burst and thus act in a feedback loop enhancing its own synthesis. Salicylic acid may also be involved in hypersensitive cell death by acting as an inhibitor of catalase, an enzyme that  
25 removes hydrogen peroxide. 4) Hydrogen peroxide may trigger production of additional defense compounds such as phytoalexins, antimicrobial low molecular weight compounds. For a review on the role of the oxidative burst and salicylic acid please see Lamb, C. and Dixon, R.A., *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 48: 251-275 (1997).

30

### Detoxification of Harvested Grain, Silage, or Contaminated Food Crop

The present invention also relates to a method of detoxifying a fumonisin or a structurally related mycotoxin with the APAO enzyme from by *Exophiala spinifera*, ATCC 74269 during the processing of grain for animal or human food consumption, during the processing of plant material for silage, or food crops contaminated with a toxin producing microbe, such as but not limited to, tomato. Since the atmospheric ammoniation of corn has proven to be an ineffective method of detoxification (see B. Fitch Haumann, *INFORM* 6:248-257 (1995)), such a methodology during processing is particularly critical where transgenic detoxification is not applicable.

In one embodiment of the present invention, fumonisin degradative enzymes are presented to grain, plant material for silage, or a contaminated food crop, or during the processing procedure, at the appropriate stages of the procedure and in amounts effective for detoxification of fumonisins and structurally related mycotoxins. Detoxification by the enzymes, the microbial strains, or an engineered microorganism can occur not only during the processing, but also any time prior or during the feeding of the grain or plant material to an animal or incorporation of the grain or food crop into a human food product, or before or during ingestion of the food crop.

Another embodiment of the present invention is the engineering of a bacterium or fungus to express the detoxification enzymes and then using the bacterium or fungus rather than the enzyme itself. There are a number of microbes that could be engineered to express the polynucleotides of the present invention. One could also activate, either inducibly or constitutively, the endogenous genes for fumonisin esterase or APAO of *Exophiala*. By overexpressing the degradative enzymes and then treating plants, seed, or silage with the microorganism, it would be possible to degrade fumonisin *in situ*.

The polynucleotides of the invention can be introduced into microorganisms that multiply on plants (epiphytes) to deliver enzymes to potential target crops. Epiphytes can be gram-positive or gram-negative bacteria, for example.

The microorganisms that have been genetically altered to contain at least one degradative polynucleotide and resulting polypeptide may be used for protecting agricultural crops and products. In one aspect of the invention, whole, i.e. unlysed, cells of the transformed organism are treated with reagents that prolong the activity of the enzyme produced in the cell when the cell is applied to the environment of a target plant. A

secretion leader may be used in combination with the gene of interest such that the resulting enzyme is secreted outside the host cell for presentation to the target plant.

The degradative enzymes can be fermented in a bacterial host and the resulting bacteria processed and used as a microbial spray. Any suitable microorganism can be used for this purpose. See, for example, Gaertner, *et al.* (1993) in *Advanced Engineered Pesticides*, (ed. Kim, Marcel Dekker, New York).

The enzymes or microorganisms can be introduced during processing in appropriate manners, for example as a wash or spray, or in dried or lyophilized form or powdered form, depending upon the nature of the milling process and/or the stage of processing at which the enzymatic treatment is carried out. See generally, Hosney, R.C., *Principles of Cereal Science and Technology*, American Assn. of Cereal Chemists, Inc., 1990 (especially Chapters 5, 6 and 7); Jones, J.M., *Food Safety*, Eagan Press, St. Paul, MN, 1992 (especially Chapters 7 and 9); and Jelen, P., *Introduction to Food Processing*, Reston Publ. Co., Reston, VA, 1985. Processed grain or silage to be used for animal feed can be treated with an effective amount of the enzymes in the form of an inoculant or probiotic additive, for example, or in any form recognized by those skilled in the art for use in animal feed. The enzymes of the present invention are expected to be particularly useful in detoxification during processing and/or in animal feed prior to its use, since the enzymes display relatively broad ranges of pH activity. The esterase from *Exophiala spinifera*, ATCC 74269, showed a range of activity from about pH 3 to about pH 6, and the esterase from the bacterium of ATCC 55552 showed a range of activity from about pH 6 to about pH 9 (US patent no. 5,716,820, *supra*). The APAO enzyme from *Exophiala spinifera* (ATCC 74269) has a pH range of activity from pH 6 to pH 9.

## **Genetic Engineering of Ruminant Microorganisms**

Ruminant microorganisms can be genetically engineered to contain and express either the fumonisin esterase enzymes or APAO isolated from *Exophiala spinifera*, ATCC 74269, or a combination of the enzymes. The genetic engineering of microorganisms is now an art recognized technique, and ruminant microorganisms so engineered can be added to feed in any art recognized manner, for example as a probiotic or inoculant. In addition, microorganisms capable of functioning as bioreactors can be engineered so as to

be capable of mass producing either the fumonisin esterases or the APAO enzyme found in *Exophiala spinifera*, ATCC 74269.

## **Use of the Fumonisin Esterase and APAO Enzymes for Detection of Reagents for Fumonisin and Related Compounds**

Another embodiment of the present invention is the use of the enzymes of the present invention as detection reagents for fumonisins and related compounds. The enzymes of the present invention can be used as detection reagents because of the high specificity of the esterase and deaminase enzymes, and the fact that hydrolysis followed by amine oxidation can be monitored by detection of hydrogen peroxide or ammonia using standard reagents (analogous to a glucose detection assay using glucose oxidase). Hydrogen peroxide is often measured by linking a hydrogen peroxide-dependent peroxidase reaction to a colored or otherwise detectable peroxidase product (e.g. Demmano, *et al.*, *European Journal of Biochemistry* 238(3): 785-789 (1996)). Ammonia can be measured using ion-specific electrodes : Fritsche, *et al.*, *Analytica Chimica Acta* 244(2): 179-182 (1991); West, *et al.*, *Analytical Chemistry* 64(5): 533-540 (1992), and all herein incorporated by reference) or by GC or other chromatographic method.

For example, recombinant or non-recombinant, active fumonisin esterase (ESP1 or BEST) and APAO proteins are added in catalytic amounts to a sample tube containing an unknown amount of fumonisins (FB1, FB2, FB3, FB4, or partial or complete hydrolysis products of these). The tube is incubated under pH and temperature conditions sufficient to convert any fumonisin in the sample to AP1, and correspondingly the AP1 to 2-OP, ammonia, and hydrogen peroxide. Then suitable reagents are added for quantification of the hydrogen peroxide or ammonia that were generated stoichiometrically from fumonisins. By comparison with control tubes that received no esterase or APAO enzyme, the amount of fumonisin present can be calculated in direct molar proportion to the hydrogen peroxide or ammonia detected, relative to a standard curve.

This invention can be better understood by reference to the following non-limiting examples. It will be appreciated by those skilled in the art that other embodiments of the invention may be practiced without departing from the spirit and the scope of the invention as herein disclosed and claimed.

## Example 1

**Fungal and bacterial isolates.** *Exophiala* isolates from maize were isolated as described in US patent no. 5,716,820, issued February 10, 1998 and pending US applications nos. 08/888,950 and 08/888,949, both filed July 7, 1997, and herein incorporated by reference.

**Isolation methods.** Direct isolation of black yeasts from seed was accomplished by plating 100 microliters of seed wash fluid onto YPD or Sabouraud agar augmented with cycloheximide (500 mg/liter) and chloramphenicol (50 mg/liter). Plates were incubated at room temperature for 7-14 days, and individual pigmented colonies that arose were counted and cultured for analysis of fumonisin-degrading ability as described in US patent no. 5,716,820, issued February 10, 1998 and pending US applications nos. 08/888,950 and 08/888,949, both filed July 7, 1997.

**Analysis of fumonisins and metabolism products.** Analytical thin-layer chromatography was carried out on 100% silanized C18 silica plates (Sigma #T-7020; 10 x 10 cm; 0.1 mm thick) by a modification of the published method of Rottinghaus (Rottinghaus, *et al.*, *J Vet Diagn Invest*, 4: 326 (1992), and herein incorporated by reference).

To analyze fumonisin esterase activity sample lanes were pre-wet with methanol to facilitate sample application. After application of from 0.1 to 2  $\mu$ l of aqueous sample, the plates were air-dried and developed in MeOH:4% KCl (3:2) or MeOH:0.2 M KOH (3:2) and then sprayed successively with 0.1 M sodium borate (pH 9.5) and fluorescamine (0.4 mg/ml in acetonitrile). Plates were air-dried and viewed under long wave UV.

For analysis of APAO activity, an alternative method was used. Equal volumes of sample and  $^{14}$ C-AP1 (1 mg/ml, pH 8, 50 mM sodium phosphate) were incubated at room temperature for one to six days. Analytical thin-layer chromatography was then carried out on C60 HPK silica gel plates (Whatman #4807-700; 10x10 cm; 0.2 mm thick). After application of from 0.1 to 2  $\mu$ l of aqueous sample, the plates were air-dried and developed in  $\text{CHCl}_3$ :MeOH: $\text{CH}_3\text{COOH}$ : $\text{H}_2\text{O}$  (55:36:8:1). Plates were then air dried, and exposed to PhosphorImager screen (Molecular Dynamics) or autoradiographic film. A Storm<sup>TM</sup> PhosphorImager (Molecular Dynamics) was used to scan the image produced on the screen.

**Alkaline hydrolysis of FB1 to AP1.** FB1 or crude fumonisin C<sub>8</sub> material was suspended in water at 10-100 mg/ml and added to an equal volume of 4 N NaOH in a screw-cap tube. The tube was sealed and incubated at 60°C for 1 hr. The hydrolysate was cooled to RT and mixed with an equal volume of ethyl acetate, centrifuged at 1000 RCF for 5 minute and the organic (upper) layer recovered. The pooled ethyl acetate layers from two successive extractions were dried under N<sub>2</sub> and resuspended in distilled H<sub>2</sub>O. The resulting material (the aminopentol of FB1 or "AP1") was analyzed by TLC.

**Enzyme activity of culture filtrate and mycelium.** *Exophiala spinifera* isolate 2141.10 was grown on YPD agar for 1 week, and conidia were harvested, suspended in sterile water, and used at 10<sup>5</sup> conidia per ml to inoculate sterile Fries mineral salts medium containing 1 mg/ml purified FB1 (Sigma Chemical Co.). After 2 weeks incubation at 28°C in the dark, cultures were filtered through 0.45 micron cellulose acetate filters, and rinsed with Fries mineral salts. Fungal mycelium was suspended in 15 mL of 0.1% FB1, pH 5.2 + 1 mM EDTA + 3 µg/mL Pepstatin A + 1.5 µg/mL Leupeptin and disrupted in a Bead Beater™ using 0.1 mm beads and one minute pulses, with ice cooling. Hyphal pieces were collected by filtering through Spin X™ (0.22 µm), and both mycelial supernatant and original culture filtrates were assayed for fumonisin modification by methods outlined above.

**Preparation of crude culture filtrate.** Agar cultures grown as above were used to inoculate YPD broth cultures (500 ml) in conical flasks at a final concentration of 10<sup>5</sup> conidia per ml culture. Cultures were incubated 5 days at 28°C without agitation and mycelia harvested by filtration through 0.45 micron filters under vacuum. The filtrate was discarded and the mycelial mat was washed and resuspended in sterile carbon-free, low mineral salts medium (1 g/liter NH<sub>3</sub>NO<sub>4</sub>; 1 g/liter NaH<sub>2</sub>PO<sub>4</sub>; 0.5 g/liter MgCl<sub>2</sub>; 0.1 g/liter NaCl; 0.13 g/liter CaCl<sub>2</sub>; 0.02 g/liter FeSO<sub>4</sub> · 7H<sub>2</sub>O, pH 4.5) containing 0.5 mg/ml alkaline hydrolyzed crude FB1. After 3-5 days at 28°C in the dark with no agitation the cultures were filtered through low protein binding 0.45 micron filters to recover the culture filtrate. Phenylmethyl sulfonyl fluoride (PMSF) was added to a concentration of 2.5 mM and the culture filtrate was concentrated using an Amicon™ YM10 membrane in a stirred cell at room temperature, and resuspended in 50 mM sodium acetate, pH 5.2 containing 10 mM CaCl<sub>2</sub>. The crude culture filtrate (approx. 200-fold concentrated) was stored at -20°C.

To obtain preparative amounts of enzyme-hydrolyzed fumonisin, 10 mg. of FB1 (Sigma) was dissolved in 20 mL of 50 mM sodium acetate at pH 5.2 + 10 mM CaCl<sub>2</sub>, and 0.25 mL of 200x concentrated crude culture filtrate of 2141.10 was added. The solution was incubated at 37°C for 14 hours, and then cooled to room temperature. The reaction mixture was brought to approx. pH 9.5 by addition of 0.4 mL of 4 N KOH, and the mixture was extracted twice with 10 mL ethyl acetate. The combined organic layers were dried under N<sub>2</sub> and resuspended in dH<sub>2</sub>O. 2.5 milligrams of organic extracted material were analyzed by Fast Atom Bombardment (FAB) mass spectrometry. The resulting mass spectrum showed a major ion at M/z (+1)=406 mass units, indicating the major product of enzymatic hydrolysis was AP1, which has a calculated molecular weight of 405.

## Example 2

### Preparation of AP1-induced and non-induced mycelium.

Liquid cultures of *Exophiala spinifera* isolate 2141.10 were prepared from YPD agar plates (Yeast Extract 10 gm, Bacto-Peptone 20 gm, Dextrose 0.5 gm, and Bacto-Agar 15 gm per liter of water). Aliquots (400-500 uL) of a water suspension of *E. spinifera* cells from YPD agar were spread uniformly onto 150 x 15 mm YPD agar plates with 4 mm sterile glass beads. The plates were incubated at room temperature for 6-7 days. The mycelia/conidia were transferred from the agar plates into Mineral Salts Medium (MSM) (Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O 0.2 gm, NH<sub>4</sub>Cl 1.0 gm, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.01 gm, FeSO<sub>4</sub>·7H<sub>2</sub>O 0.02 gm per liter of distilled water, pH 4.5) and centrifuged at 5000 x g, 4°C, 20 minutes to pellet the cells. The cell pellet was rinsed once in 40 mL MSM and recentrifuged. The rinsed cell pellet was used to inoculate MSM at a 1:19 ratio of packed cells: MSM. The culture to be induced was supplemented with AP1 to a final concentration of 0.5-1.0 mg/ml and incubated at 28°C, 100 rpm, in the dark to induce catabolic enzymes. The non-induced cultures did not receive AP1 but were placed on media containing 4-ABA at the same concentration as AP1. The supernatants were removed by filtration through 0.45 cellulose acetate. The remaining mycelial mat was washed with sterile MSM and then frozen in liquid nitrogen for storage.

### Example 3

#### Effect of FB1 and AP1 on maize coleoptiles

Maize coleoptiles from 4 day dark-grown germinated maize seeds were excised above the growing point and placed in 96-well microtiter plates in the presence of 60 microliters of sterile distilled water containing FB1 or AP1 at approximately equimolar concentrations of 1.5, .5, .15, .05, .015, .005, .0015, or .0005 millimolar, along with water controls. After 2 days in the dark at 28° C the coleoptiles were placed in the light and incubated another 3 days. Injury or lack thereof was evaluated as follows:

	0	.0005	.0015	.005	.015	.05	.15	.5	1.5	mM
FB1	-	-	-	-	+/-	+	+	+	+	
AP1	-	-	-	-	-	-	-	-	+	

+ = brown necrotic discoloration of coleoptile

- = no symptoms (same as water control)

The results indicate there is at least a 30-fold difference in toxicity between FB1 and AP1 to maize coleoptiles of this genotype. This is in general agreement with other studies where the toxicity of the two compounds was compared for plant tissues: In *Lemna* tissues, AP1 was approx. 40-fold less toxic (Vesonder *et al.*, "Arch Environ Contam Toxicol 23: 464-467 (1992).). Studies with both AAL toxin and FB1 in tomato also indicate the hydrolyzed version of the molecule is much less toxic (Gilchrist *et al.*, *Mycopathologia* 117: 57-64 (1992)). Lamprecht *et al.* also observed an approximate 100-fold reduction in toxicity to tomato by AP1 versus FB1 (Lamprecht *et al.*, *Phytopathology* 84: 383391 (1994)).

### Example 4

#### Effect of FB1 and AP1 on maize tissue cultured cells (Black Mexican Sweet, BMS)

FB1 or AP1 at various concentrations was added to suspensions of BMS cells growing in liquid culture medium in 96-well polystyrene plates. After 1 week the cell density in wells was observed under low power magnification and growth of toxin-treated wells was compared to control wells that received water. Growth of BMS cells was significantly inhibited at 0.4 micromolar FB1, but no inhibition was observed until 40 micromolar AP1. This represents an approximate 100-fold difference in toxicity to maize



tissue cultured cells. Similarly Van Asch et al. (VanAsch *et al.*, *Phytopathology* 82: 1330-1332 (1992)) observed significant inhibition of maize callus grown on solid medium at 1.4 micromolar FB1. AP1 was not tested in that study, however.

### Example 5

#### APAO Activity

A cell-free extract that contains the amine oxidase activity was obtained by subjecting substrate-induced *Exophiala spinifera* cells to disruption using a Bead Beater™ in 50 mM Na-phosphate, pH 8.0, and recovering the cell-free supernatant by centrifugation and .45 micron filtration. Catabolic activity is assayed by incubating extracts with AP1 (hydrolyzed fumonisin B1 backbone) or <sup>14</sup>C-labelled AP1 with the extract and evaluating by TLC on C18 or C60 silica. The product 2-OP has a lower R<sub>f</sub> than AP1 and is detected either by radiolabel scan or by H<sub>2</sub>SO<sub>4</sub> spray/charring of the TLC plate. 2-OP does not react with the amine reagent fluorescamine that is routinely used to detect AP1 on TLC plates, suggesting that the amine group is missing or chemically modified. Activity is greater at 37°C than at room temperature, but following 30 min. at 65°C or 100°C no AP1 catabolic activity remained. Activity is maximal at pH 9. At pH 9, complete conversion to 2-OP occurred in 30 minutes. Activity is retained by 30,000 dalton molecular weight cutoff membrane, but only partially retained by 100,000 dalton molecular weight cutoff membrane. Other amine-containing substrates were tested for modification by the crude extract. Fumonisin (with tricarballic acids attached) is not modified by the extract, indicating that ester-hydrolysis must occur first for the APAO to be able to be effective in modifying FB1. Other long-chain bases (sphingosine, sphinganine, and phytosphingosine) are apparently not modified by the crude APAO, suggesting the enzyme(s) is specific for the fumonisin backbone. Preparative amounts of the product, named 2-OP, have also been purified and analyzed by C13 nmr. The results indicate that 2-OP has a keto group at carbon 2 instead of an amine, consistent with an oxidative deamination by an amine oxidase. The C13 nmr data also indicate that 2-OP spontaneously forms an internal hemiketal between C-1 and C-5, resulting in a 5-membered ring with a new chiral center at C-2. All other carbon assignments are as in AP1, thus 2-OP is a compound of composition C<sub>22</sub>H<sub>44</sub>O<sub>6</sub>, FW 404. The product of the enzyme acting on hydrolyzed fumonisin would not be expected to display any significant toxicity.

Other enzymes were tested for their ability to modify AP1. All enzymes were assayed by radiolabeled TLC, as described above, under optimal conditions at 37<sup>0</sup> Celsius, overnight or longer. The results are as follows:

Deaminating	EC	Source	Result
Monoamine Oxidase	1.4.3.4	bovine plasma	negative
D-amino oxidase	1.4.3.3	porcine kidney; TypeX	negative
L-amino oxidase	1.4.3.2	C.adamanteus venom; TypeI	negative
Tyramine oxidase	1.4.3.4	Arthrobacter spp	negative
Methylamine dehydrogenase	1.4.99.3	Paracoccus denitrificans	negative
Aralkyl amine dehydrogenase	1.4.99.4	Alcaligenes faecalis	negative
Phenylalanine ammonia lyase	4.3.1.5	Rhodotorula glutinis; TypeI	negative
Histidine ammonia lyase	4.3.1.3	Pseudomonas fluorescens	negative
L-aspartase	4.3.1.1	Hafnia alvei (Bacterium cadaveris)	negative
Tyrosine oxidase	1.14.18.1	mushroom	negative
Lysine oxidase	1.4.3.14	Trichoderma viride	negative
Diamine oxidase	1.4.3.6	porcine kidney	negative

The results were negative for each enzyme tested. Therefore isolates from the American Type Culture Collection (ATCC) were collected. The ATCC isolates selected were listed as containing amine-modifying enzymes or were capable of growth/utilization on amine-containing substrates. The isolates were tested to determine if they could grow on or utilize AP1 as the sole nitrogen source and if any could modify AP1 to a new compound(s). The nitrogen sources that were used in liquid cultures were AP1 0.1% (w/v), s-butylamine 0.1% (v/v), n-butylamine 0.1% (v/v), and ammonium nitrate 0.2% (w/v). These were prepared in Vogel's Minimal Media (without NH<sub>4</sub>NO<sub>3</sub>) containing 2% sucrose. The isolates were inoculated into the various media and monitored for growth over 2-3 weeks. They were also assayed with the <sup>14</sup>C-radiolabeled TLC assay for AP1 modification. In summary, none of the isolates tested exhibited modification of AP1 *in vivo*. Clearly the APAO enzyme from *Exophiala spinifera* is unique and unusual in its ability to modify the AP1 toxin.

## EXAMPLE 6

### Isolation of the trAPAO Polynucleotide

The trAPAO polynucleotide was identified using a proprietary transcript imaging method that compares transcript patterns in two samples and allows cloning of differentially expressed fragments. This technology was developed by CuraGen® (New Haven, CT). (see Published PCT patent application no. WO 97/15690, published May 1,

1997, and hereby incorporated by reference) Fluorescently-tagged, PCR amplified cDNA fragments representing expressed transcripts can be visualized as bands or peaks on a gel tracing, and the cDNA from differentially expressed (induced or suppressed) bands can be recovered from a duplicate gel, cloned and sequenced. Known cDNAs can be identified without the need for cloning, by matching the predicted size and partially known sequence of specific bands on the tracing.

In the present invention two RNA samples were obtained from cultures of *E. spinifera* grown for a specified period in a mineral salts medium containing either AP1 (induced condition), or gamma-aminobutyric acid (ABA; non-induced condition) as a sole carbon source. In the induced condition, fumonisin esterase and APAO enzyme activities are detected, whereas in the non-induced condition these activities are not detected. The methods used for induction of APAO and detection of activity are described earlier (see Example 2 and Example 5). RNA was extracted from induced mycelium by Tri-Reagent methods (Molecular Research Center Inc., Cincinnati, Ohio) only grinding a frozen slurry of tissue and Tri-Reagent with a mortar and pestle until almost melted and adding an additional extraction after the phase separation by extracting the aqueous phase one time with phenol, and two times with a phenol:chloroform:isoamyl alcohol mixture. The RNA's were submitted for CuraGen® transcript imaging to detect cDNA fragments that are induced specifically in the presence AP1. In the resulting gel tracing several bands were found which showed induction of at least 2-fold up to 79-fold or even 100-fold or more in AP1. In the resulting gel tracing several bands were found which showed induction of at least 10-fold in AP1-grown cells as compared to cells grown in ABA. The sequence of two highly induced bands can be found in Table 1.

**TABLE 1**

**Nucleotide sequence of two CuraGen® bands that were identified as strongly induced by AP1 in cultures of *Exophiala spinifera*.**

>k0n0-395.5\_b (SEQ ID NO: 1)  
GGGCCCCGGCGTTCTCGTAGGCTGCGCGGAGTTGGTCCCAGACAGACTTTTGTCTGCTACCTGCTTG  
GACTGTTGGGACCACTTCCGTCCCGGGTCTCCGACCATGAAACAGGTAATGGACCATTGTCTGAT  
CGACGTCGATGCTGGTATCTCTGGCAAATGAGATGGGGTCACAGCTCGATTGGAGGACGCCCCGA  
GAAGCCTTGTTTCGCGCCACCACGGCTTGTTCCCATACGAAGACTATCTTGCTATAGTAGCCCAGG  
ATAGAATTTTCCGCCAATGCTTGCTTCTCGGCGGGAAGAGGTGGTGAAAATGTCAAGGTGGGAT  
ACAAGGTTGTCGGTAACGAAACCANCACCTTTTGTCTTCGGAACACGGCGC

>r0c0-182.3\_6 (SEQ ID NO: 2)  
GAATTTTCCGCCAATGCTTGCTTCTCGGCGGGAAGAGGTGGTGAAAATGTCAAGGTGGGATACA  
AGGTTGTCGGTAACGAAACCACCACCTTTTGTCTCGGAACACGGCGCCCGAGGCCGATCGTAC  
TGTACAGCCGGATGCCGACTGCTCAATTTACGCGACGGGGGTGTTGAGGTGCAC

5

Two of the highly induced bands, k0n0-395.5, and r0c0-182.3 showed significant sequence homology to a family of enzymes, flavin-containing amine oxidases (EC 1.4.3.4), that oxidize primary amines to an aldehyde or ketone, releasing ammonia and

10

TABLE 2

15 Identification of a putative flavin amine oxidase from *E. spinifera*: AP1-induced transcript fragments with amine oxidase homology. BLAST 2.0 default parameters.

Clone ID	Size	Best Hit	Best Hit Name, source	Prob	from	to	Likely function
k0n0-395.5	395 bp	P40974	putrescine oxidase, <i>Micrococcus rubens</i> , EC 1.4.3.10 Length = 478	8.0 e-07	276	333	oxidation of C-2 amine of AP1
r0c0-182.3 (contigs with k0n0-395)	182 bp	P12398	monoamine oxidase type A (MAO-A) [ <i>Bos taurus</i> ] Length = 527	0.0039	238	296	oxidation of C-2 amine of AP1

20 The chemical structure of the primary product of AP1 deamination is thought to be a 2-keto compound which cyclizes to a hemiketal at carbons 2 and 5. Therefore it is predicted that this induced enzyme is responsible for deamination of AP1.

Using sequence derived from k0n0-395.5, a partial cDNA was obtained by 3' and 5' RACE-PCR (Chenchik, *et al.*, *CLONTECHniques* X 1:5-8 (1995); Chenchik, *et al.*, A new method for full-length cDNA cloning by PCR. In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis*. Ed. Krieg, P.A. (Wiley-Liss, Inc.), 273-321 (1996)). A RACE cloning kit from CLONTECH was used, to obtain the RACE amplicons. Briefly, poly A+ RNA is transcribed to make first strand cDNA using a "lock-docking" poly T, cDNA synthesis primer, the second strand is synthesized and the

25

Marathon cDNA adaptor is ligated to both ends of the ds cDNA. Diluted template is then used with the Marathon adapter primer and in separate reactions either a 5' Gene Specific Primer (GSP) or a 3'GSP is used to produce the 3' or 5' RACE amplicon. After characterization of the RACE product(s) and sequencing, full-length cDNAs may be generated by 1) end-to-end PCR using distal 5' and 3' GSPs with the adapter-ligated ds cDNA as template, or 2) the cloned 5' and 3'-RACE fragments may be digested with a restriction enzyme that cuts uniquely in the region of overlap, the fragments isolated and ligated. Subsequently, the RACE-generated full-length cDNAs from 1) and 2) may be cloned into a suitable vector.

In combination with the supplied adapter primer the following gene specific primers were used: for 3' RACE the oligonucleotide N21965: 5'-

TGGTTTCGTTACCGACAACCTTGATCCCC-3' (SEQ ID NO: 3) and for 5' RACE, the oligonucleotide N21968: 5'-GAGTTGGTCCCAGACAGACTTTTGTCGT-3' (SEQ ID NO: 4).

The nucleotide sequence of the trAPAO polynucleotide, k0n0-395\_6.5, from *Exophiala spinifera* is shown in SEQ ID NO: 5. The polypeptide sequence of trAPAO is shown in SEQ ID NO: 6.

A second clone of APAO containing an unspliced intron was also found. The polynucleotide sequence of trAPAO-I polynucleotide, k0n0-395\_5.4, the intron containing clone, from *Exophiala spinifera*, can be found in SEQ ID NO: 7. The polypeptide sequence of trAPAO-I with the intron spliced out is shown in SEQ ID NO: 8. The polypeptide sequence of trAPAO-I without the intron spliced out is shown in SEQ ID NO: 9.

## EXAMPLE 7

### Heterologous Expression of trAPAO

Protein alignments generated with PileUp (GCG) indicate that k0n0-395\_6.5 (trAPAO) is similar in size to other flavin amine oxidases and is close to being full length with respect to the amino terminus of their class of proteins. The k0n0-395\_6.5 sequence contains a complete  $\beta$ - $\alpha$ - $\beta$  fold that is required for dinucleotide (FAD) binding, close to the amino end. The k0n0-395 sequence appears to lack only a variable amino terminal segment that varies in length from 5 amino acids in rat monoamine oxidases A & B to 40 amino acids in length in *Aspergillus* MAO-N. The function of these amino terminal extensions is not known; they are not recognizable as secretion signals. Based on the likely

localization of the *Exophiala* APAO outside the cell membrane, the prediction is that k0n0-395 would have a signal sequence similar to that of the fumonisin esterase cloned from the same organism (US patent no. 5,716,820, *supra*). Using GenomeWalker™, it is possible to clone the 5' end of the transcript and upstream genomic regulatory elements.

5 However, the signal sequence is not expected to be critical to the functionality of the enzyme; in fact, the preferred strategy for heterologous expression in maize and *Pichia pastoris* involves replacing the endogenous signal sequence (if present) with an optimized signal sequence for the organism, e.g. barley alpha amylase for maize and the yeast alpha factor secretion signal for *Pichia*. In maize transformed with fumonisin esterase, the  
10 barley alpha amylase signal sequence gave higher amounts of functional protein than the native fungal signal, therefore replacement of the native fungal signal sequence is a logical optimization step. Since many of the amine oxidases have a positively charged amino acid near the N-terminus and upstream of the dinucleotide binding site, an additional optimization step included adding a codon for the lysine (K) to the N-terminus of the  
15 trAPAO clone (k0n0-395\_6.5, SEQ ID NO: 5). This clone is designated K:trAPAO and can be seen in SEQ ID NOS: 10 and 11. The extra lysine is at amino acid 1 and nucleotides 1-3.

## EXAMPLE 8

### 20 *Pichia* Expression of trAPAO

For optimum expression of trAPAO in *Pichia pastoris* the alpha mating factor signal peptide was fused in-frame with K:trAPAO coding sequence and can be seen in SEQ ID NOS: 16 and 17. The nucleotide sequence of clone pPicZalphaA:K:trAPAO contains a PCR-amplified insert comprising the k0n0-395 open reading frame with an additional  
25 lysine residue at the amino terminus, with a 5' EcoRI site and 3' NotI site for in-frame cloning into the alpha factor secretion vector pPicZalphaA. Nucleotides 1-267 contain the yeast  $\alpha$  mating factor secretion signal. The amino acid sequence of shown in SEQ ID NO: 17 contains the trAPAO polypeptide produced from pPicZalphaA:K:trAPAO following transformation into *Pichia pastoris*.

30 For cloning into the *Pichia pastoris* expression vector, two cloning strategies were used. The cDNA k0n0-395\_5.4 was generated by using end-to-end PCR using distal 5' and 3' GSPs with the adapter-ligated ds cDNA as template. pPicZalphaA, distal

oligonucleotide primers were designed with 5' restriction enzyme sites that contain a 23-25 bp anchored overlap of the 5' end (sense strand) and 3' end (antisense strand) for cloning into the open reading frame of k0n0-395; the 3' primer also included the stop codon. The primer sequences are N23256: 5'-ggggaattcAAAGACAACGTTGCGGACGTGGTAG-3' (SEQ ID NO: 12) and N23259: 5'-ggggcgccgcCTATGCTGCTGGCACCAGGCTAG-3' (SEQ ID NO: 13). A second method was used to generate k0n0-395\_6.5. 5' RACE and 3' RACE products were generated using a distal primer containing the necessary restriction enzyme sites, stop codon, etc as described above and paired with a "medial" GSP. The "medial primers" N21965: 5'-TGGTTTCGTTACCGACAACCTTGATCCCC-3' (SEQ ID NO: 14) for 3' RACE and for 5' race, the oligonucleotide N21968: 5'-GAGTTGGTCCCAGACAGACTTTTGTCGT-3' (SEQ ID NO: 15). Adapter-ligated ds cDNA was used as template. The isolated 5' and 3'-RACE fragments were digested with a restriction enzyme that cuts uniquely in the region of overlap, in this case Bgl I, isolated and ligated into the expression vector. The digestible restriction sites allow cloning of the inserts in-frame into EcoRI/NotI digested pPicZalphaA. pPicZalphaA is an *E. coli* compatible *Pichia* expression vector containing a functional yeast alpha factor secretion signal and peptide processing sites, allowing high efficiency, inducible secretion into the culture medium of *Pichia*. The resulting 1.4 kb bands were cloned into EcoRI/NotI digested pPicZalphaA plasmid.

SEQ ID NO: 16 contains the polynucleotide sequence of clone pPicZalphaA:K:trAPAO, a PCR-amplified insert that comprises the k0n0-395 open reading frame with an additional lysine residue at the amino terminus, and a 5' EcoRI site and 3' NotI site for in-frame cloning into the alpha factor secretion vector pPicZalphaA. SEQ ID NO: 17 contains the amino acid sequence of the trAPAO polypeptide produced from pPicZalphaA:K:trAPAO following transformation into *Pichia pastoris*. The alpha factor secretion signal and a lysine are added.

*Pichia* was transformed as described in Invitrogen Manual, Easy Select™ *Pichia* Expression Kit, Version B, #161219, with the trAPAO polynucleotide as described above with either an intron (trAPAO-I, negative control, no expression of active trAPAO since *Pichia* does not splice introns very efficiently) or without an intron (capable of making an active APAO protein). The *Pichia* culture fluids and pellets were assayed for APAO activity as described earlier.

The set of frozen six day *Pichia* culture cell pellets contained two samples with intron (SEQ ID NO: 7) in gene construct, # 11, # 14, and two samples without intron in gene construct (SEQ ID NO: 5), #6, # 52. The six day culture fluids from the same cultures were used to spike with crude fungal enzyme for positive controls.

5        The 50 µl cell pellets were resuspended in 150 µl cold 50mM Na-phosphate, pH 8.0, and divided into two fresh 500 µl tubes. One tube was kept on ice with no treatment, the pellet suspension, and one tube was used for lysis. An equal volume of 0.1 mm zirconia-silica beads was added to each tube. The tubes were BeadBeat™ for 15 seconds then cooled on ice 5 minutes. This was repeated three times. The crude lysate was then  
10       transferred to another tube for assay or lysate suspension.

      The TLC assays were performed as follows, the samples are 1) pellet suspensions; 10 µl; 2) lysate suspensions; 10 µl; 3) media controls-mixed 5 µl media with 5 µl crude fungal enzyme; 10 µl; 4) positive control-used crude fungal enzyme undiluted; 10 µl; 5) substrate control-used 50mM Na-phosphate, pH8.0; 10 µl. Ten microliters of each sample  
15       plus 10 µl of <sup>14</sup>C-AP1 (1 mg/ml, 50 mM Na-phosphate, pH 8) was incubated at room temperature for 6 days. One microliter of the sample was spotted onto C18 and C60 TLC plates. The C18 plates were developed in MeOH:4% KCl (3:2). The C60 plates were developed in CHCl<sub>3</sub>:MeOH:CH<sub>3</sub>COOH:H<sub>2</sub>O (55:36:8:1). The plates were then air dried and then exposed to a PhosphorScreen™ for 2-3 days. A Storm™ PhosphorImager was  
20       used to develop the images.

      A positive TLC result is obtained if an additional radioactive spot appears at a lower R<sub>f</sub> of the produced AP1 modification earlier identified as 2-OP, a deaminated product of AP1. In samples # 6 and # 52 (without intron) the AP1-modifying enzyme activity (conversion of AP1 to 2-OP) was detected in pellet suspensions and pellet lysates,  
25       although the majority of activity was associated with the pellet suspensions. In samples #11 and #14 (with intron) a minimal amount of AP1-modifying enzyme activity was detectable in the pellet lysate of # 14 only, which indicates *Pichia* cannot process the intron efficiently.

      This experiment verified APAO activity can be detected in *Pichia* transformants,  
30       which verifies that trAPAO as described functions correctly in degrading AP1. The activity is associated with cell suspensions, which show higher activity than pellet lysates.



Pellet lysates may show less activity due to release of endogenous proteases during lysis of the cells.

## EXAMPLE 9

### Expression of trAPAO in *E. coli*

The vector for expressing K:trAPAO in *E. coli* is pGEX-4T-1. This vector is a prokaryotic glutathione S-transferase (GST) fusion vector for inducible, high-level intracellular expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST. GST gene fusion vectors include the following features, a lac promoter for inducible, high-level expression; an internal lac Iq gene for use in any *E. coli* host; and the thrombin factor Xa or PreScission Protease recognition sites for cleaving the desired protein from the fusion product. The insert of interest, k0n0-395\_6.5 (K:trAPAO), was subcloned into the 5' EcoRI site and a 3' NotI site allowing in-frame expression of the GST:K:trAPAO fusion peptide. The polynucleotide sequence of the GST:K:trAPAO fusion can be found in SEQ ID NO: 18. The GST fusion with polylinker can be found at nucleotides 1 to 687. The K:trAPAO can be found at nucleotides 688 to 2076. The resulting polypeptide for the GST:K:trAPAO fusion can be seen at SEQ ID NO: 19. Amino acids 1 to 229 represent the GST fusion plus polylinker and amino acids 230 to 692 represent the K:trAPAO portion of the fusion.

*E. coli* was transformed with the pGEX-4T-1 vector containing K:trAPAO as described in BRL catalogue, Life Technologies, Inc. catalogue; Hanahan, D., *J. Mol. Biol.* 166:557 (1983) Jessee, J. *Focus* 6:4 (1984); King, P.V. and Blakesley, R., *Focus* 8:1, 1 (1986), and hereby incorporated by reference. The transformed *E. coli* was induced by addition of IPTG (isopropyl b-D-thiogalactopyranoside). Four samples of soluble extract and four samples of insoluble inclusion bodies were tested for trAPAO activity as described in Example 9. APAO activity was present in all soluble samples and two insoluble samples. Highest activity was found at 10 uM IPTG induction. Thus the pGEX-4T-1 vector containing k0n0-395\_6.5 construct is capable of producing active APAO enzyme in *E. coli*.

## EXAMPLE 10

### The Complete Nucleotide Sequence of the *Exophiala* APAO Gene

Using Genome Walker, the complete nucleotide sequence of the *Exophiala* APAO gene was recovered. The nucleotide sequence described in SEQ ID NO: 5 is missing a  
5 portion of the 5' end of the native gene. The missing portion of the 5' end of the native gene is not necessary for expression of an active APAO enzyme, as can be seen in Examples 9 and 10. The complete nucleotide sequence of APAO can be seen in SEQ ID NO: 22. The translation of SEQ ID NO: 22 can be found in SEQ ID NO: 23.

## EXAMPLE 11

### Expression of APAO and ESP1 in transgenic maize callus

One of the preferred constructs for expression in maize is the nucleotide sequence of the trAPAO fused to the barley alpha amylase signal sequence. The nucleotide sequence of K:trAPAO translational fusion with barley alpha amylase signal sequence, for  
15 expression and secretion of the mature trAPAO in maize can be seen in SEQ ID NO: 20. Nucleotides 1-72, represent the barley alpha amylase signal sequence; nucleotides 73-75, represent the added lysine residue; and nucleotides 76 -1464 , represent the trAPAO cDNA. The amino acid sequence translation of SEQ ID NO: 20 can be found in SEQ ID NO: 21. Amino acids 1 to 24 represent the barley alpha amylase signal sequence and  
20 amino acids 25 to 463 is the sequence of K:trAPAO.

Maize embryos were transformed with linear DNA (insert, lacking a bacterial antibiotic resistance marker), derived from constructs containing three transcription units: 1) a PAT selectable marker gene (Wohlleben *et al.*, *Gene* 70, 25-37 (1988)), 2) fumonisin esterase ESP1 fused to a barley alpha amylase signal sequence, and 3) full length APAO  
25 without or with an amino-terminal barley alpha amylase signal sequence, (P13603, comprising a PAT selectable marker fused to a 35S promoter, fumonisin esterase ESP1 fused to a barley alpha amylase signal sequence and the ubiquitin promoter, and APAO fused to the ubiquitin promoter and P13611, comprising a PAT selectable marker fused to the 35S promoter, fumonisin esterase ESP1 fused to a barley alpha amylase signal  
30 sequence and the ubiquitin promoter and APAO fused to a barley alpha amylase signal sequence and the ubiquitin promoter). In these constructs both ESP1 and APAO were linked to the maize ubiquitin promoter and first intron. In a third construct, the same three

transcriptional units were cloned into an Agrobacterium T1 vector (P15258, the construct comprises a PAT selectable marker, fumonisin esterase ESP1 fused to a barley alpha amylase signal sequence and APAO). Stably transformed callus or T0 plants regenerated from callus were tested for ESP1 and APAO activity in buffer extracts of leaf tissue, using  
5 radiolabeled FB1 and/or AP1 and C18 thin-layer chromatography. Positive controls consist of non-transformed tissue spiked with *E coli*-expressed recombinant ESP1 or APAO. The results indicate that both ESP1 and APAO activities can be detected in transgenic maize callus and plants.

#### 10 Expression of ESP1 and APAO in transgenic callus

Construct	Sample ID Number	ESP1 activity (TLC)	APAO activity (TLC)
13603	3065.031-2	+	+
13603	3065.034-3	+	+
13603	3065.1117-3	+	+
13603	3065.11s7-n13	+	+
13603	3065.117-2	+	+
13603	3065.1115-2	+	+
13603	3065.1115-6	+	+
13603	3065.1112-1	+	+
13603	3065.118-6	+	+
13603	3065.11s3-1	+	+
13603	3065.11s1-13	+	+
13603	2805.762-2	+	+
13603	3065.1110-2	+	+
13603	3065.039-2	+	+
13611	3065.293-3	+	+
13611	3065.263-1	+	+
13611	3070.24.2.3	+	+

Transgenic plants were regenerated from the transgenic callus positive for both ESP1 and APAO activity by standard methods known in the art. Enzyme activity was  
15 tested as described previously. As can be seen below transgenic maize plants can successfully express both ESP1 and APAO enzymes.

#### Expression of APAO and ESP1 in transgenic maize plants (T0)

Construct	Sample ID Number	ESP1 activity (TLC)	APAO activity (TLC)
13603	910080	+	+
13603	910081	+	+
13603	917065	+	+

Another preferred construct for expression of APAO in a plant is targeting the APAO to the peroxisome. Maize embryos were bombarded with insert containing APAO operably linked to ubiquitin promoter and a peroxisomal targeting sequence (Gould, *et al.*, *J Cell Biol* 108:1657-1664 (1989)); ESP1 operably linked to ubiquitin promoter and the  
5 barley alpha amylase signal sequence; and a selectable marker of PAT operably linked to the 35S promoter (construct number I14952). Negative controls were unbombarded embryos/callus. Positive controls were unbombarded embryos/callus spiked with purified enzyme. Transformed callus was then tested for ESP1 or APAO activity as previously described. Out of 67 samples tested 18 samples contained both ESP1 activity and APAO  
10 activity. Peroxisomally targeted APAO and apoplast targeted fumonisin esterase can both be successfully expressed in a plant cell.

Another preferred construct for expression of APAO in a plant is targeting the APAO to the mitochondrial membrane. A C-terminal extension is required for targeting monoamine oxidases MAO-A and MAO-B to mammalian outer mitochondrial  
15 membranes. An MAO-A, MAO-B, or functionally similar C-terminal extension can be fused in-frame to APAO or trAPAO to facilitate localization of this enzyme to the mitochondrial membrane of maize or other transformed species.

## EXAMPLE 12

### Comparison of APAO Sequence With Other Sequences

The *Exophiala* cDNA of APAO (SEQ ID NO: 22) contains an 1800 bp open reading frame coding for a 600 amino acid polypeptide (SEQ ID NO: 23) with divergent  
25 homology to two classes of proteins. The carboxy three-fourths of APAO (amino acids 137 to 593) is strongly homologous to flavin amine oxidases, a group of enzymes catalyzing the oxidative deamination of primary amines at carbon 1. The amine oxidase function of the carboxy terminal domain was confirmed by expression of a truncated APAO polypeptide (from 137 to 600) in both *Pichia pastoris* and *E. coli*, using AP1 as a  
30 substrate (see Example 9). The amino terminal portion of APAO, in contrast, (from approx. 5 to 134) shows significant homology to a group of small deduced open reading frames (ORFs) reported in several bacteria and blue-green algae, as well as several higher organisms. These ORFs code for small proteins of unknown function, ranging in size from

14 to 17 kDA. The juxtaposition of these divergent homologies in a single polypeptide has not been reported previously.

Flavin amine oxidases (E.C. 4.1.4.3) are a group of flavoenzymes found in both higher and lower organisms, and serve a variety of functions in catabolism. They catalyze the oxidative deamination of primary amino groups located at the C-1 position of a variety of substrates, resulting in an aldehyde product plus ammonia and hydrogen peroxide. The APAO enzyme described in this report is the first flavin amine oxidase known to attack a primary amine not located at C-1 (i.e. C-2 of AP1) and resulting in a keto rather than aldehydic product. However, amino acid oxidases, while not closely related to flavin amine oxidases, are flavoenzymes that oxidize a C-2 amine adjacent to a C-1 carboxyl group.

The monoamine oxidases MAO A & B, (from human, bovine, and trout), are localized in the mitochondrial outer membrane of higher organisms and regulate the level of neurotransmitters. Microbial examples include a fungal amine oxidase (*Aspergillus niger* (niger) MAO-N) involved in amine catabolism, and a bacterial putrescine oxidase from a gram (+) bacterium (*Micrococcus rubens*.). The primary polypeptides vary in length from 478 to 527 amino acids, and share regions of high amino acid sequence conservation at the 5' end as well as at various points through the coding region. Protein alignments generated with PileUp (GCG) indicate that trAPAO contains all conserved domains found in this class of proteins including those near the 5' end.

The amine oxidase domain of trAPAO contains several key features shared by this class of enzymes, including an amino-terminal dinucleotide (ADP) binding region characterized by a beta-alpha-beta stretch containing three invariant glycines (G -X-G-X-X-G) in the beta-alpha turn. In trAPAO, this sequence is (DVVVVGAGLSG). This region is involved in FAD binding. Absent are several features unique to the mammalian amine oxidases, including several essential cysteine residues (Wu *et al.*, *Mol Pharm* 43:888 (1993)), one of which (Cys-406 of MAO-A) is involved in covalent binding of FAD, and a carboxy-terminal extension that has been demonstrated to be involved in transporting to and anchoring the MAO in the outer mitochondrial membrane. The *Aspergillus* enzyme MAO-N has been demonstrated to contain non-covalent FAD, and also lacks the conserved cysteine. Therefore it is possible that the *Exophiala* APAO enzyme has a non-covalent FAD. The *Aspergillus* MAO-N has a carboxy-terminal

tripeptide Ala-Arg-Leu that is involved in peroxisomal targeting and localization; this sequence is absent from *Exophiala* APAO.

The amine oxidase domain of trAPAO contains a total of seven cysteines, compared to ten for the *Aspergillus* enzyme and only two for the *Micrococcus* enzyme.

- 5 The mammalian MAO enzymes contain variable numbers of cysteines (at least ten), some of which are highly conserved (including the FAD binding residue mentioned above). The trAPAO sequence also has two putative glycosylation sites (NDS, NQS) towards the amino end.

- 10 The purpose of the amino-terminal extension of APAO and the basis for its homology to a group of 14-17 kDa proteins is not clear. In *Synechocystis*, a similar polypeptide ORF is located immediately upstream of the NADP-dependent glutamine dehydrogenase (gdhA) and has been shown to be required for functional expression of gdhA (Chavez et al, 1995). However, in trAPAO the domain is clearly not necessary for enzymatic activity, as shown by the results of the expression experiments using the
- 15 truncated APAO. An interesting clue comes from the frequent association of this small ORF with gene clusters involved in oxidoreductase activity in bacteria, or induced by heat stress in mice, suggesting a possible role in redox protection. A byproduct of amine oxidase activity is hydrogen peroxide. Flavoenzymes and other redox enzymes are often susceptible to inactivation by hydrogen peroxide (Schrader *et al.*, *App Microb Biotechnol*
- 20 45:458; Aguirre, *et al.*, *J Bacteriol* 171:6243 (1989)), and it is possible that this protein has a protective role against oxidants such as hydrogen peroxide. Alternatively, this domain could be involved in enzyme function, localization or association of the enzyme with other structures. No signal peptide region can be detected in this amino terminal region.

- In multiple sequence alignment using GCG PileUp, trAPAO is most similar to
- 25 putrescine oxidase of *Micrococcus rubens*, Swissprot accession number P40974, (30% identical amino acids, 40% similar). Homology with several mammalian monoamine oxidases A and B, Swissprot accession numbers P21397 (*Homo Sapiens* mao a), P19643 (*Rattus norvegicus* mao b), P21396 (*Rattus norvegicus* mao a), and P21398 (*Bos taurus* mao a), is somewhat less, ranging from 25 to 28% identity and 36 to 40% similarity.
- 30 Homology to the only other fungal flavin amine oxidase known, MAO-N from *Aspergillus niger* (Swissprot accession number P46882), is somewhat lower (24% identical, 34%

similar). The microbial enzymes are considerably divergent from each other, while the mammalian monoamine oxidases share 65 to 87% identity.

The amino terminal domain (ATD) of APAO also shows homology to a 14.5 kD protein from human and rat phagocytes that shows translational inhibition activity *in vitro* (Swissprot accession # P52758, P52759) Schmiedeknecht, *et al.*, *Eur J Biochem* 242 (2), 339-351 (1996)), and includes a heat-responsive protein from mouse (Samuel, *et al.*, *Hepatology* 25 (5), 1213-1222 (1997)). This suggests that this family of proteins is involved in regulating cellular metabolism. No example exists in which this domain is fused to a larger protein domain, however, making APAO unique. Without intending to be limited by theory, all of this suggests, that this domain plays a regulatory role in APAO gene expression, possibly to prevent translation of the message when it is not needed. This raises the question of how translation of the message is restored when active enzyme is required by the *Exophiala* cell. Possibly there are alternative start sites that begin downstream of the inhibitor domain; or proteolysis, complexing, degradation, or phosphorylation/dephosphorylation of the inhibitor domain when it is not needed. The first possibility is less likely because there are no other ATG codons prior to the ATG at 122-124 that constitutes the predicted start site of APAO. The second possibility cannot be easily tested, although there is a casein kinase site in the ATD. Alternative roles for the ATD include oligomerization of the APAO protein, or anchoring the protein to some intracellular site, such as the membrane.

A parallel example of regulatory control over another flavoenzyme, human flavin monooxygenase 4 (FMO-4), by a C-terminal extension has been reported (Itagaki, *et al.*, *J of Biol Chem* 271(33): 20102-20107 (1996)). In this case the introduction of a stop codon prior to the 81 base C-terminal extension allowed expression of active enzyme in heterologous systems. The role of the C-terminal portion was not elucidated, however. In another example, alternative splicing led to a shorter gene product that complexed with and interfered with the function of the normally spliced version (Quinet, *et al.*, *J of Biol Chem* 268(23): 16891-16894 (1993)). In another case, an alternative splicing-generated insert in another protein led to inhibition of cell growth (Bhat, *et al.*, *Protein Engineering* 9(8): 713-718 (1996)). In yet another variation, fas/Apo1 splicing variants prevent apoptosis, apparently through a 49 amino acid domain shared by all variants ((Papoff, *et al.*, *J of Immunology* 156(12): 4622-4630 (1996)).

### EXAMPLE 13

#### **Making a fusion protein containing fumonisin esterase and AP amine oxidase activity in the same polypeptide.**

5           The enzyme activities of fumonisin esterase and AP amine oxidase can be combined in a single polypeptide by using the open reading frames together either with or without a spacer region between the two polypeptides. This creates a hybrid protein with dual enzyme activities that can be exported as a unit to the apoplast, and will allow both enzyme activities to be conveniently localized to the same area of the cell wall. The two  
10   cDNA's can be combined in either order, but the preferred method is to link them in the order NH<sub>3</sub>-Esterase:Amine Oxidase-COOH. The spacer, if present, may consist of a short stretch of amino acids such as GGGSGGGS, or a set of amino acids that comprises a protease cleavage site that can be acted on by an apoplastic protease. This would result in the production of stoichiometric amounts of both esterase and APAO enzymes in the  
15   apoplast.

          The esterase-APAO fusion protein can be made with either the fumonisin esterase from *E. spinifera* 2141.19 (ESP1) or fumonisin esterase from bacterium 2412.1 (BEST1). Since the pH range for maximum activity of BEST1 is similar to that of APAO (range 6.0 to 8.0), these may present the most effective combination in fusion form. As described in  
20   previous examples these fusion sequences can be placed in the appropriate expression vectors and used to express proteins in either bacteria or plants.

          The nucleotide sequence of ESP1 contains three nucleotide differences and three corresponding amino acid differences for the ESP1 sequence disclosed in pending US applications nos. 08/888,950 and 08/888,949, both filed July 7, 1997. Both the sequences  
25   disclosed in the present application and the sequences disclosed in the pending US applications contain functional fumonisin esterase genes. For the purposes of the present invention, either the original ESP1 sequences or the ESP1 sequences disclosed may be used in combination with the APAO sequences or in fusion sequences. The nucleotide sequence of a BAA:ESP1:K:trAPAO construct for plant expression can be found in SEQ  
30   ID NO: 24 and the translation in SEQ ID NO: 25. The nucleotide sequence for a BAA:BEST1:K:trAPAO construct for plant expression can be found in SEQ ID NO: 26 and the translation in SEQ ID NO: 27. The nucleotide sequence of a GST:ESP1:K:trAPAO fusion for bacterial expression in a pGEX-4T-1 or similar vector



can be found in SEQ ID NO: 28 and the translation in SEQ ID NO: 29. The nucleotide sequence for a GST:BEST1:K:trAPAO fusion for bacterial expression in a pGEX-4T-1 or similar vector can be seen in SEQ ID NO: 30 and the translation in SEQ ID NO: 31.

5

## EXAMPLE 14

### APAO Substrate Studies

The following assay was used to determine the substrate specificity of the APAO enzyme. Reaction mix: 436  $\mu$ l of 200 mM Na-phosphate, pH8.0; 50  $\mu$ l substrate (10 mM); 2  $\mu$ l Amplex Red (1 mg in 200  $\mu$ l DMSO); and 2  $\mu$ l of Peroxidase (5000 U/ml). The APAO enzyme was recombinant enzyme produced as GST fusion in *E. coli*, purified over a glutathione affinity column and cleaved with thrombin to remove the GST. All components were mixed at room temperature. The initial rate was determined in a spectrophotometer at 572 nm over one minute by absorbance units/second (BLANK). Ten microliters of APAO at 70 ug/ml was added and mixed. The initial rate was again determined at 572 nm over one minute in absorbance units/second (SAMPLE). The rates were converted to absorbance units/minute. The BLANK value was subtracted from the SAMPLE value. The absorbance units were converted to  $\mu$ M  $H_2O_2$  wherein 1  $\mu$ M  $H_2O_2$  equals 0.138 absorbance units at pH 8.0.

### SUBSTRATES FOR APAO

SUBSTRATE	RATE $\mu$ M $H_2O_2$ /min
1 mM Fumonisin B1	0.1429
1 mM AP1	0.8876
0.5mg/mL Fumonisin B2	0.3058
1 mM Fumonisin B3	0.1449
0.5mg/mL Fumonisin B4	0.1728
1 mM norepinephrine	0.0087
1 mM epinephrine	0.0071
1 mM dopamine	0.0040
1 mM spermine	0.0002

25 **NOT SUBSTRATES FOR APAO** (defined as compounds resulting in less than 1% conversion to hydrogen peroxide by APAO relative to AP1 under similar conditions of time, pH, temperature, and substrate concentration): 2-phenylethylamine, spermidine,

EDTA-Na<sub>2</sub>, tryptamine, putrescine, benzamidine, serotonin, cadaverine, Pefabloc SC, tyramine, 1,3-diaminopropane, leupeptin, histamine, hydroxylamine, aprotinin, deprenyl, Fumonisin C4, isoniazid, sphingosine, phenelzine, sphinganine, phytosphingosine, D-alanine, DL-alanine, L-arginine, L-asparagine, L-aspartic acid, D-aspartic acid, L-cysteine, 5 L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, DL-lysine, L-methionine, DL-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, L-tyrosine, L-valine.

10

## EXAMPLE 15

### Removal of glycosylation sites from APAO.

Some cytosolic enzymes, when engineered for secretion by fusion with a heterologous signal peptide, lack function due to glycosylation at one or more potential glycosylation sites (amino acid consensus sequence N-X-S/T) that are not normally 15 glycosylated in the native environment (Farrell LB, Beachy RN, *Plant Mol Biol* 15(6):821-5 (1990)). Since APAO lacks a recognizable signal sequence, it may be cytoplasmically localized in *Exophiala spinifera*, although secretion by some other method not involving a signal peptide cannot be ruled out. APAO contains two potential glycosylation sites, which can potentially be glycosylated, when APAO is secreted in a plant or other 20 eukaryotic cell. These glycosylation sites can be eliminated without affecting protein function by site-directed mutagenesis using standard protocols (such as kits available from CLONTECH Laboratories, Inc. (Palo Alto, CA)).

SEQ ID NO: 33 shows the amino acid sequence of a GST:APAO in which two amino acids of APAO have been changed by site-directed mutagenesis to eliminate two 25 potential glycosylation sites. The polynucleotide sequence of SEQ ID NO: 33 can be found in SEQ ID NO: 32. The first mutation changes asparagine at amino acid 201 of APAO to serine, and the second mutation changes serine at amino acid 206 of APAO to asparagine. Other mutations at either amino acid 200, 201, 202, 203, 204, 205, 206, or 207 of APAO, or a combination of these, can also be engineered to accomplish the removal of 30 the glycosylation signal (Mellquist, J.L., Kasturi, L., Spitalnik, S.L., and Shakin-Eshelman, S.H., 1998. The amino acid following an Asn-X-Ser/Thr sequence is an important determinant of n-linked core glycosylation efficiency. *Biochemistry* 37:6833).

Other modifications to APAO can be made to improve its expression in a plant system, including site-directed mutagenesis to remove selected cysteine residues, which may be detrimental to proper folding when the protein is secreted into the endomembrane system for delivery to the apoplast. Cysteines are present at residues 64, 109, 167, 292, 351, 359, 387, 461, and 482, and may or may not be involved in disulfide crosslinking in mature, folded APAO. Using standard methods of site-directed mutagenesis, one or more of these residues can be substituted with alanine or other suitable amino acid, resulting in a modified version of APAO that retains its activity and specificity but displays better activity and stability in an extracellular environment. It is possible that one or more cysteines is involved in covalent attachment of the FAD moiety to the APAO protein, and elimination of this cysteine would be expected to reduce or abolish activity.

All publications and patent applications in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference.

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention